Simone Barreira Morais

Development and Application of Electroanalytical Procedures in in Vitro

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DEVELOPMENT AND APPLICATION OF ELECTROANALYTICAL PROCEDURES IN IN VITRO CYTOCOMPATIBILITY STUDIES OF 316L STAINLESS STEEL

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO
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DEVELOPMENT AND APPLICATION OF

ELECTROANALYTICAL PROCEDURES IN \textit{IN VITRO}

CYTOCOMPATIBILITY STUDIES OF 316L STAINLESS STEEL

Simone Barreira Morais

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Publications

This thesis is based on the publications numbered below from 1 to 9. In the publications numbered from 1 to 6, S. Morais was responsible for all the experimental work except in the paper 6 where the DRIFT analyses were performed by H. T. Gomes and Professor J. L. Faria; the studies undertaken in papers 7 and 8 were carried out in collaboration with the Biomaterials Research Group from the University of Leiden and all the experimental work was done by Professor G. S. Carvalho and S. Morais; in the study 9, N. Dias helped with the cellular cultures and S. Morais was responsible for all the restant work.

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International Journals


3- S. Morais, G. S. Carvalho, J. P. Sousa, "Nickel Determination in Osteoblast-Like Cell Culture Medium by Adsorptive Cathodic Stripping Voltammetry with a Mercury Microelectrode", Electroanalysis 1997, 9, 422.
4- S. Morais, G. S. Carvalho, J. P. Sousa, "Chromium Determination in Osteoblast-Like Cell Culture Medium by Catalytic Cathodic Stripping Voltammetry with a Mercury Microelectrode", submitted.

5- S. Morais, G. S. Carvalho, J. P. Sousa, "Potentiometric Determination of Total and Ionized Calcium in Osteoblast-like Cell Culture Medium", Electroanalysis 1996, 8, 1174.


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RESUMO

O principal objectivo desta dissertação consistiu no desenvolvimento e aplicação de técnicas electroanalíticas em estudos de citocompatibilidade específica, com o intuito de avaliar in vitro as interacções existentes na interface osso-implante entre os produtos de corrosão libertados do aço inoxidável AISI 316L, aquando da sua utilização como implante ortopédico, e as células ósseas, bem como caracterizar o processo de mineralização.

Técnicas de voltametria de redisolução com adsorção foram desenvolvidas e optimizadas, recorrendo ao uso de um microeléctrodo revestido com um filme de mercúrio, para a quantificação de ferro, crómio e níquel em concentrações da ordem das partes por bilhão, em meios de cultura recolhidos de testes de citocompatibilidade efectuados in vitro com células derivadas de medula óssea de coelho, rato e homem. Estas células foram cultivadas, em condições experimentais que favorecem a proliferação e diferenciação de osteoblastos, e expostas a várias concentrações de suspensões metálicas contendo os produtos da dissolução electroquímica do aço inoxidável AISI 316L, bem como os seus componentes majoritários (ferro, crómio e níquel) preparados a partir dos sais.

Neste trabalho, foram também desenvolvidos métodos potenciométricos, baseados em padrões que simulam o meio de cultura dez ou duas vezes diluído, para a determinação, respectivamente, de cálcio total e ionizado que, combinados com a
técnicas de espectrofotometria (esta técnica foi usada para monitorizar a variação da concentração de fósforo, no meio de cultura, com o tempo nos ensaios de biocompatibilidade), ajudaram a avaliar o processo de mineralização.

A associação dos resultados da análise dos meios de cultura (relativa, nomeadamente, à quantificação de ferro, crómio, níquel, cálcio total e ionizado, bem como fósforo) com os obtidos pelos ensaios morfológicos, histopatológicos e bioquímicos possibilitou a caracterização dos efeitos provocados por diferentes doses de produtos de corrosão na morfologia, proliferação, diferenciação e função de osteoblastos derivados da medula óssea de coelho, rato e homem. Os resultados obtidos indicaram que a inibição do metabolismo, as reacções de toxicidade e alterações da estrutura e síntese do tecido podem, de facto, ocorrer na presença de produtos de corrosão, bem como, geralmente, a severidade da resposta celular aumenta à medida que aumenta a concentração das suspensões metálicas às quais as células estão expostas. Outra observação interessante verificada foi a diminuição da concentração de crómio (com exceção do crómio no estado de oxidação trivalente) e níquel no meio de cultura na presença de células de rato e de homem. Estes elementos parecem ser, provavelmente, os responsáveis pela toxicidade dos produtos de corrosão do aço inoxidável AISI 316L para as concentrações subletais investigadas (i.e. \(8.95 \times 10^{-6}\) mol/L Fe + \(2.35 \times 10^{-6}\) mol/L Cr + \(1.72 \times 10^{-6}\) mol/L Ni e \(8.78 \times 10^{-6}\) mol/L Fe + \(4.31 \times 10^{-6}\) mol/L Cr + \(2.56 \times 10^{-6}\) mol/L Ni).
RÉSUMÉ

L' objectif principal de ce travail fut de développer et appliquer des procédés électroanalytiques à la caractérisation de tests de cytotocompatibilité conçus pour évaluer in vitro les interactions entre les ions métalliques, libérés pendant la corrosion d'un implant orthopédique d'acier inoxydable 316L, et les cellules osseuses ainsi qu'étudier le processus de minéralisation.

Des méthodes de voltampérométrie inverse avec adsorption, utilisant une microélectrode dont la surface est revêtue d'un film de mercure, ont été spécialement adaptées pour mesurer des quantités très faibles de fer, de chrome et de nickel dans des échantillons de milieux de culture parvenant de tests de cytotocompatibilité réalisés in vitro avec des cellules de moelle osseuse prélevées chez le lapin, le rat et l'homme. Ces cellules furent cultivées dans des conditions expérimentales qui aident à la prolifération et différenciation des ostéoblastes, et exposées à des différentes concentrations de suspensions métalliques contenant les produits de corrosion obtenus par dissolution électrochimique de l'acier inoxydable 316L ou ces principaux ions métalliques préparés séparément à partir des sels.

Dans cette étude, des méthodes potentiométriques directes, se servant de solutions standard ayant une composition ionique similaire à celle du milieu de culture dilué dix fois (pour la détermination de la concentration totale de calcium) ou deux fois (pour la
détermination de la concentration ionique de calcium), ont été développées pour mesurer la concentration de calcium. L’application de celles-ci et de procédures spectrophotométriques ont aidé à caractériser ce qui se passe quand la minéralisation se donne dans des conditions normales, c’est-à-dire, sans l’influence d’interférences ou dans la présence de produits de corrosion.

L’association des résultats obtenus par les analyses chimiques du milieu de culture (concernant notamment le fer, le chrome, le nickel ainsi que la concentration totale et ionique de calcium et de phosphore), par les essais morphologiques, les histochimiques et les biochimiques ont permis d’étudier les effets provoqués par des diverses doses de produits de corrosion sur la morphologie, la prolifération, la différenciation et les fonctions des ostéoblastes de lapin, de rat et de l’homme. Les expériences menées ont démontré que les ions métalliques sont capables d’induire des réactions toxiques, l’altération de la synthèse et de la structure des tissus, l’inhibition du métabolisme, et aussi que, dans la grande majorité des cas, la détérioration cellulaire s’aggrave lorsqu’augmente la concentration des produits de dégradation en contact avec les cellules. Par ailleurs, il fut observée une diminution des taux de chrome (avec l’exception du chrome ayant la valence plus trois) et de nickel dans les milieux de culture des essais réalisés avec des cellules de rat et d’homme. Ces éléments paraissent être les responsables pour la toxicité induite par les produits de corrosion de l’acier inoxydable 316L aux concentrations étudiées (c’est-à-dire 8.95x10⁻⁶ mol/L Fe + 2.35x10⁻⁶ mol/L Cr + 1.72x10⁻⁶ mol/L Ni et 8.78x10⁻⁶ mol/L Fe + 4.31x10⁻⁶ mol/L Cr + 2.56x10⁻⁶ mol/L Ni).
SUMMARY

The main goal of the present work was to develop and apply suitable electroanalytical procedures to characterise cytocompatibility tests envisaged to evaluate \textit{in vitro} the interactions of the major metal ions that are released during the corrosion of a 316L stainless steel orthopaedic implant with bone cells at the tissue-implant interface, and the mineralisation process.

Adsorptive stripping voltammetric procedures, using a mercury film microelectrode, were optimised to quantify down to the part per billion level iron, chromium and nickel in samples of incubation medium collected from rabbit, rat and human bone marrow derived cells, cultured in experimental conditions that favour the proliferation and differentiation of osteoblastic cells, and exposed to different concentrations of 316L stainless steel corrosion products and the corresponding major separate metal ions.

Direct potentiometric procedures for total and ionised calcium determination were developed based on standards which simulated the osteoblast-like cell culture medium ten-fold (total calcium quantification) or two-fold (ionised calcium quantification) diluted, and applied in combination with spectrophotometry (this technique was used to monitor the alteration of the phosphorus concentration in the cell culture medium throughout the incubation time of the \textit{in vitro} biocompatibility tests) to help to characterise the mineralisation process in cultures grown in control conditions and exposed to metallic slurries.
The combination of the chemical analyses of the incubation media (concerning namely, iron, chromium, nickel, total and ionised calcium and phosphorus) with morphological (observation by light microscopy, scanning electron microscopy coupled with X-ray microanalysis as well as transmission electron microscopy), histochemical (identification of alkaline phosphatase positive cells and calcium and phosphates deposits) and biochemical (enzymatic reduction of MTT, alkaline phosphatase activity, DNA content and total protein content) assays provided means to shed some light on the dose- and time-effects of the degradation products on rabbit, rat and human osteoblast-like cells morphology, proliferation, differentiation and function. The results obtained indicated that inhibition of normal metabolism, toxic reactions and alterations of tissue synthesis and structure can occur in the presence of corrosion products and also that, generally, the severity of the cellular response increases as the concentration of the metallic slurries at which cells were exposed increased. Interestingly, nickel and chromium (not the species in the trivalent state) were found to be retained by cells and appear, probably, to be the elements responsible for the toxicity of stainless steel corrosion products at the sublethal concentrations (i.e. $8.95 \times 10^{-6}$ mol/L Fe + $2.35 \times 10^{-6}$ mol/L Cr + $1.72 \times 10^{-6}$ mol/L Ni and $8.78 \times 10^{-6}$ mol/L Fe + $4.31 \times 10^{-6}$ mol/L Cr + $2.56 \times 10^{-6}$ mol/L Ni) studied.
I- GENERAL INTRODUCTION
BIOCOMPATIBILITY AND BIOMATERIALS

A definition of biocompatibility, arrived at by the consensus of experts, is: "...the ability of a material to perform with an appropriate host response, in a specific application"\(^1\).

This statement implies a two-way interaction since biocompatibility refers to\(^1\):

(a) a collection of processes involving different but interdependent mechanisms of interaction between the material and the tissue;

(b) the ability of the material to perform a function which depends not only on the intrinsic mechanical and physical properties of the material but also on its interaction with the tissues;

(c) the appropriate host responses. It does not stipulate that there should be no response, but rather that the response should be appropriate or acceptable in view of the function that has to be performed;

(d) the specific application. Biocompatibility of a material always has to be described with reference to the situation in which it is used. No material is unequivocally biocompatible: many materials may be biocompatible under one or more defined conditions but cannot be assumed to display biocompatibility under all conditions.
Biocompatibility is not an intrinsic material property and cannot be considered as such.

Biomaterials are composed of established series of metals, ceramics and glasses, polymers and composites. Table 1 lists examples of their widespread clinical applications and Table 2 provides estimates of the number of biomaterial devices used nowadays in clinical settings evidencing the importance of the biomaterial market. This century has seen an explosion in the number and the varieties of surgical implants used in the treatment of various medical problems. The increase in the number of implants has been accompanied by an increase in the life expectancy of patients and a decrease in the average age of patients receiving an implant.
### Table 1: Examples of commonly used biomaterials and their applications

<table>
<thead>
<tr>
<th>Material category</th>
<th>Material</th>
<th>Principal applications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metals</strong></td>
<td>316 stainless steel</td>
<td>bone and joint replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spinal instrumentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fracture fixation</td>
</tr>
<tr>
<td></td>
<td>Ti, Ti-Al-V, Ti-Al-Nb</td>
<td>bone and joint replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fracture fixation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dental implants</td>
</tr>
<tr>
<td></td>
<td>Co-Cr alloys</td>
<td>pacemaker encapsulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bone and joint replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dental implants and restorations</td>
</tr>
<tr>
<td></td>
<td>Au alloys</td>
<td>heart valves</td>
</tr>
<tr>
<td></td>
<td>Ag products</td>
<td>dental restorations</td>
</tr>
<tr>
<td></td>
<td>Hg-Ag-Sn amalgam</td>
<td>antibacterial agents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dental restorations</td>
</tr>
<tr>
<td><strong>Ceramics and glasses</strong></td>
<td>alumina</td>
<td>joint replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dental implants</td>
</tr>
<tr>
<td></td>
<td>zirconia</td>
<td>joint replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bone repair and augmentation</td>
</tr>
<tr>
<td></td>
<td>calcium phosphates</td>
<td>surface coatings on metals</td>
</tr>
<tr>
<td></td>
<td>bioactive glasses</td>
<td>bone replacement</td>
</tr>
<tr>
<td></td>
<td>porcelain</td>
<td>dental restorations</td>
</tr>
<tr>
<td></td>
<td>carbons</td>
<td>percutaneous devices</td>
</tr>
<tr>
<td></td>
<td></td>
<td>coatings for blood-contacting devices</td>
</tr>
<tr>
<td><strong>Polymers</strong></td>
<td>polyethylene</td>
<td>joint replacement</td>
</tr>
<tr>
<td></td>
<td>polypropylene</td>
<td>sutures</td>
</tr>
<tr>
<td></td>
<td>polytetrafluoroethylene</td>
<td>soft-tissue augmentation</td>
</tr>
<tr>
<td></td>
<td>polyesters</td>
<td>vascular prostheses</td>
</tr>
<tr>
<td></td>
<td>polyurethanes</td>
<td>vascular prostheses</td>
</tr>
<tr>
<td></td>
<td>polymethyl methacrylate</td>
<td>drug delivery systems</td>
</tr>
<tr>
<td></td>
<td></td>
<td>blood-contacting devices</td>
</tr>
<tr>
<td></td>
<td>silicones</td>
<td>dental restorations</td>
</tr>
<tr>
<td></td>
<td>hydrogels</td>
<td>intraocular lenses</td>
</tr>
<tr>
<td></td>
<td></td>
<td>joint replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>soft-tissue replacement</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ophthalmology</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ophthalmology blood-contacting devices</td>
</tr>
<tr>
<td><strong>Composites</strong></td>
<td>dimethacrylate resin-quartz</td>
<td>dental restorations</td>
</tr>
<tr>
<td></td>
<td>carbon fiber-thermosetting resins</td>
<td>bone repair</td>
</tr>
<tr>
<td></td>
<td>carbon fiber-thermoplastics</td>
<td>bone repair</td>
</tr>
<tr>
<td></td>
<td>carbon-carbon</td>
<td>bone and joint replacement</td>
</tr>
</tbody>
</table>
Table 2- Selected biomedical implant applications\(^2\): magnitude of use\(^a\).

<table>
<thead>
<tr>
<th>Application</th>
<th>Numbers used per year</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ophthalmologic</strong></td>
<td></td>
</tr>
<tr>
<td>Intraocular lenses</td>
<td>1400000</td>
</tr>
<tr>
<td>Contact lenses</td>
<td>250000000(^b)</td>
</tr>
<tr>
<td>Retinal surgery implants</td>
<td>50000</td>
</tr>
<tr>
<td>Prostheses after enucleation</td>
<td>5000</td>
</tr>
<tr>
<td><strong>Cardiovascular</strong></td>
<td></td>
</tr>
<tr>
<td>Vascular grafts</td>
<td>350000</td>
</tr>
<tr>
<td>Arteriovenous shunts</td>
<td>150000</td>
</tr>
<tr>
<td>Heart valves</td>
<td>75000</td>
</tr>
<tr>
<td>Pacemakers</td>
<td>130000</td>
</tr>
<tr>
<td>Blood bags</td>
<td>300000000</td>
</tr>
<tr>
<td><strong>Reconstructive</strong></td>
<td></td>
</tr>
<tr>
<td>Breast prostheses</td>
<td>100000</td>
</tr>
<tr>
<td>Nose, chin</td>
<td>10000</td>
</tr>
<tr>
<td>Penile</td>
<td>40000</td>
</tr>
<tr>
<td>Dental</td>
<td>20000</td>
</tr>
<tr>
<td><strong>Orthopaedic</strong></td>
<td></td>
</tr>
<tr>
<td>Hips</td>
<td>90000</td>
</tr>
<tr>
<td>Knees</td>
<td>65000</td>
</tr>
<tr>
<td>Shoulders, finger joints</td>
<td>50000</td>
</tr>
<tr>
<td><strong>Other devices</strong></td>
<td></td>
</tr>
<tr>
<td>Ventricular shunts</td>
<td>21500</td>
</tr>
<tr>
<td>Catheters</td>
<td>200000000</td>
</tr>
<tr>
<td>Oxygenators</td>
<td>50000</td>
</tr>
<tr>
<td>Renal dialysers</td>
<td>16000000</td>
</tr>
<tr>
<td>Wound drains</td>
<td>300000</td>
</tr>
<tr>
<td>Sutures</td>
<td>20000000</td>
</tr>
</tbody>
</table>

\(^a\)Approximate annual usage in United States
\(^b\)Worldwide
Metals enjoy a broad popularity in orthopaedics due mainly to the following reasons:\(^4\):

(a) metals have high elastic *moduli* and reasonable yield points such that structures may be constructed that will bear significant loads without large elastic deformations or any permanent deformation;

(b) metals have high enough ductility and stresses that exceed the yield point produce plastic deformation rather than sudden brittle fracture, permitting measures to be taken to modify use or to replace components before loss of integrity results;

(c) metals also possess sufficient plasticity to have fatigue endurance limits, thus suiting them for designs required to withstand great numbers of load-unload cycles, such as bone plates or proximal femoral components;

(d) metals may be fabricated into parts by a wide variety of conventional techniques and, in most cases, may have their mechanical properties adjusted before the final shape is attained;

(e) when care is taken in fabrication, surface finishing, and handling, metal devices have good to excellent resistance to the variety of external and internal environments encountered in orthopaedic practice.
The orthopaedic alloys most extensively used are 316L stainless steel, cobalt-chromium alloys and titanium and its alloys. The properties of these biomaterials are sensitive to differences in processing, however, as a general guide, Table 3 presents relative alloy system ratings with respect to some principal design considerations. Austenitic stainless steels combined moderate yield and ultimate strengths with high ductility. This last characteristics and, in particular, the significant retention of ductility after large amounts of cold work, associated with relatively low cost, render them attractive for use as fixation devices after bone fracture.

Table 3- Comparison of properties of orthopaedic alloys.

<table>
<thead>
<tr>
<th>Property</th>
<th>Stainless steel</th>
<th>Co-base</th>
<th>Ti-base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modulus</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Yield strength</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ultimate strength</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Endurance limit</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Ductility</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Machinability</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Corrosion resistance</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Cost</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*Key: 1=highest, 4=lowest. When the same number appears more than once in a row, ranges overlap.*
Interactions between the metallic biomaterial and the biological environment

Numerous complex events occur upon insertion of a foreign material into a bloody wound site, which are dependent on factors such as the implant site\textsuperscript{9-10}; material properties and surface treatment\textsuperscript{1,4,11-12}; implant design; surgical variables; as well as the condition of the patient\textsuperscript{13-15}. The relativity of biocompatibility is clearly evidenced which brings to mind Williams admonition that the unique circumstances pertaining to individual cases have to be considered when defining biocompatibility\textsuperscript{1,16}. However, from a simplicist point of view, the multifactorial variety of processes governing the biological milieu/biomaterial interactions may be considered a sequence of three steps, namely, initial interfacial reactions (1); effects of the tissue environment on the biomaterial (2), and host response to the biomaterial (3).

1- Initial interfacial reactions

There is ample evidence that an implanted device will quickly adsorb a monolayer containing many proteins (over than 200 proteins exist in plasma)\textsuperscript{2} and consequently, the cells that arrive at the surface after the protein adsorption process has commenced will no longer interface directly with the material but rather with the material covered by a layer\textsuperscript{1}. With soft (muscle and skin) and hard (bone, joints and teeth) tissues, the role of the protein layer has not been elucidated\textsuperscript{1,17-18}, but it is likely to be important in
determining the nature of cell-material interactions since it will be cell-protein rather than cell-substrate interfaces that will be established.

2- Effects of the tissue environment on the metallic orthopaedic biomaterial

Orthopaedic implants may suffer chemical/electrochemical attack or/and mechanical damage through interaction with the body resulting in the release of metal as ions or particles. Mechanical degradation primarily involves direct overload or cyclic fatigue and in most cases occurs coupled with chemical/electrochemical attack (i.e. corrosion). Stainless steel is well known to have relatively high corrosion resistance (although lower than Co-Cr alloys and Ti and Ti alloys) because of a self-passivating mechanism and a formation of a stable chromium oxy-hydroxide film on the surface. This passivated layer limits the corrosion being the overall metal ion release reduced (it will be proportional to the corrosion rate in the passive state). A method of improving the corrosion resistance of stainless steels, Co-Cr and Ti alloys consists in immersing them in HNO₃ solutions (this procedure is routinely done nowadays for metallic implants). For stainless steels, this treatment improves the pitting potential, dissolve the surface sulphide inclusions (which are normally the most susceptible sites for attack) and at the same time increases the amount of Cr in the film. The corrosion potential in the human body lies usually in the passivity region. However, the aggressivity of the biological environment, determined mainly by the presence of chloride ions (0.11 mol/L in interstitial fluid; chloride ions severely limit the extent of the passive range of stainless steels), dissolved gases and reactive species formed during metabolic events (acting either as corrosion stimulators or inhibitors), in combination
with the variation of the oxidising power of the environment that occurs during the healing process (inflammatory responses and local infection are especially productive of low pH, complex organic acids and reactive oxygen species such as hydrogen peroxide\textsuperscript{4,28,34}) may promote the falling of the corrosion potential to the active dissolution region or to the pitting region\textsuperscript{28}. Although the appearance of corrosion does not necessarily imply implant failure, it is clear that implanted stainless steels are unable to resist at all forms of corrosion attack that can occur in the bioenvironment\textsuperscript{13}, which are:

(a) general corrosion

It consists in the uniform dissolution of a metal when exposed to the bulk solution\textsuperscript{4,13}. This process was particularly important in the earlier days of internal fixation when low resistance types of stainless steels were used\textsuperscript{19,28} and does not represent a problem anymore for the mechanical integrity of currently implanted stainless steels\textsuperscript{19,28,36-37}.

(b) galvanic corrosion

It arises when two metals, having different corrosion potentials, are in contact in an electrolyte, producing an electron flow between them\textsuperscript{4,13}. Different corrosion potentials may be generated by metals or alloys of differing composition or by the same nominal alloy with differing metallurgical state\textsuperscript{13} (e.g., zones of different crystalline structures; work hardening and surface-finishing processes that produce plastic deformation generally make the deformed metal basic with respect to undeformed material of the same composition\textsuperscript{4}). The corrosion rate of the more electronegative metal or portion of the device is generally increased (relative to its
isolated corrosion rate$^{13}$) and depends on the relative sizes of the different metals, the nature of the direct contact$^4$, etc. As a good general policy it is advisable to avoid combination of dissimilar metals$^4$. However, the only absolute contraindication appears, from several published data$^{28,37}$, to be the use of stainless steel as one component.

(c) crevice corrosion

This type of corrosion is not a function of the material but of the geometry of the implant$^{38}$. Various implant designs imply small gaps or crevices, like screw head-plates hole junctions, articulation surfaces of prostheses, and hip screw-plate junctions$^{19}$, that produce a restricted chemical exchange between the electrolyte in the crevice and the bulk electrolyte leading to oxygen depletion (oxygen is reduced within the crevice without sufficient replenishment from the bulk electrolyte) and acidification (hydrolysis of the metal chlorides produces insoluble hydroxides and free HCl)$^{13}$. Increasing acidity and chloride ion concentration will generally accelerate the corrosion rate and corrosion within the crevice becomes autocatalytic$^{13,19}$.

(d) pitting corrosion

Pitting is a localised form of corrosion that arises from a defect in the passive film, promoted by oxygen depletion, concentration of damaging species, scratches, handling damage or structural irregularities, and results in the formation of tunnels or pits in the metal$^{19}$. Once a pit is initiated in stainless steel, in the physiological milieu, an active-passive cell is established with a potential difference of 0.5 to
0.6 V$^{39}$ leading to a high corrosion rate. The mechanism of pitting corrosion proceeds in a very similar manner as the one for crevice corrosion$^{4,13-14,19}$. Pits are a hazard in highly stressed devices as they constitute points of stress concentration and may serve as initiation points for fatigue failures$^4$.

(e) intergranular corrosion

This type of attack occurs preferentially in chloride solutions at the grain boundaries$^{14}$. Stainless steels, if improperly heat treated after fabrication, may corrode by this mechanism owing to a relative depletion of chromium from the regions near the grain boundaries$^4$. This phenomenon is called sensitisation$^4$ and is clearly avoidable in light of the available metallurgical techniques.

(f) erosion and fretting corrosion

These closely related forms of corrosion are due to motion between the surrounding electrolyte and the metallic surface -erosion corrosion- and between a metal and another solid body -fretting corrosion. The former, in extreme cases, may actually erode the passive layer but generally serves to increase the attack by other mechanisms sweeping the reaction products away and providing new amounts of dissolved reactants, such as chloride ions and oxygen$^4$. When the physical removal or disruption of the protective oxide film occurs continuously provoked by fretting and repassivation does not follow quickly enough, corrosion starts$^{19}$. The intensity of wear depends on load, relative motion, surface configuration, hardness wear resistance of the material and combinations of materials$^{19}$. Electrochemical considerations indicate that stainless steel is most unsuitable for metal-metal
prosthesis due to the amount of wear debris produced\textsuperscript{40}. Also, through fretting conditions for pitting and crevice corrosion may be created due to the alteration of the surface configuration of the implant leading to the formation of small cavities where body fluid can be trapped\textsuperscript{19}.

\textbf{(g) stress corrosion and corrosion fatigue}

Stress corrosion and corrosion fatigue are caused by the conjoint action of a corrosive environment, such as body fluids, and tensile stress or cyclic stress, respectively. Cracks may appear on the metal propagating until fracture occurs\textsuperscript{13-14,19}.

Corrosion is more severe in multipart devices, such as plate and screw combinations, than in one part devices\textsuperscript{4} and, as demonstrated by several studies, the majority of cases of corrosion is due to crevice or/and fretting corrosion\textsuperscript{19,41-46} (accounted for ca. 90\%\textsuperscript{41-42}). Nowadays, various surface treatments, such as ion implantation and coatings, are being studied and applied in order to decrease degradation, by shielding the metal from the biological milieu, or/and selectively guide the biological response to the implant\textsuperscript{47-50}. Ceramics eliminate the problem of metallic corrosion and, especially, aluminum oxide offers the best alternative in total hip replacement due to its very high resistance to wear and low friction\textsuperscript{51}.

\textbf{3- Host response to the metallic orthopaedic biomaterial}

Host response is defined as "the reaction of a living system to the presence of a material"\textsuperscript{52}. Reactions to the implant and its corrosion products can involve tissues
adjacent to or in the vicinity of the device -local host response- as well as those remote from the site of implantation -systemic and remote host response\textsuperscript{1,4}.

3.1- \textbf{Local host response}

The initial disruption of local blood flow by the fracture causes haemorrhage, cell death and an inflammatory response (reaction of the body to tissue injury or infection)\textsuperscript{53-56}. The inflammatory process is aimed at eliminating, or at least containing, the causative agent such that the tissue can be subsequently repaired and remodeled\textsuperscript{1}. Fig. 1 shows the cellular progression that is expected in healing tissues and the development of stiffness and strength of the healing tissues\textsuperscript{4}.

![Diagram](image)

\textbf{Figure 1}- Cell populations and mechanical properties of a healing wound\textsuperscript{4}. 
The inflammatory phase involves activation of the coagulation cascade, the inflammatory cell response (polymorphonuclear leukocytes accumulate followed by infiltration of macrophages and lymphocytes) and the recruitment and proliferation of undifferentiated mesenchymal cells originating the granulation tissue\textsuperscript{1,56}. This tissue, which surrounds the fracture site, forms the foundation for the successive healing process by degrading devitalised tissue and providing chondrogenic and osteogenic precursor cells that proliferate and differentiate to serve their specific functions in the fracture repair process\textsuperscript{56}. The result of chondrogenesis is cartilage expansion which bridges the fracture site and provides the mechanical stability needed for osteogenesis \textit{i.e.}, bone formation\textsuperscript{56} (in this case endochondral ossification). Active bone-forming cells, osteoblasts, are highly polarised mononuclear cells that are responsible for the synthesis and secretion of the organic component of the extracellular matrix of new bone, a substance known as osteoid which subsequently mineralises by the deposition of hydroxyapatite crystals in it to form bone (\textit{ca.} 90\% collagens + proteoglycans + glycoproteins + \textit{g}-carboxy glutamic acid containing proteins + enzymes + growth factors + proteolipids)\textsuperscript{57-59}. Some of the osteoblasts remain free at the surface of the existing matrix, while others become trapped in the hard matrix and are gradually converted into osteocytes\textsuperscript{59}. Osteocytes are connected with each other, surface osteoblasts and osteogenic cells by intercellular processes lying in \textit{canaliculi} (Fig. 2)\textsuperscript{58-59}. Although osteocytes do not divide, they are nevertheless active and may contribute to the transport of mineral\textsuperscript{58}. Despite the extracellular location of biological mineralisation, it is a cell regulated event and products of osteoblasts, such as alkaline phosphatase, are known to play a role\textsuperscript{57}. 
Figure 2: Deposition of bone matrix by osteoblasts.

During the reparation phase and meanwhile the process of endochondral ossification occurs, new bone is formed by appositional growth, intramembranous ossification, which does not require cartilage intermediate. Several factors, such as the rigidity of fracture fixation, hormonal and nutritional status, degree of trauma, bone loss, vascular injury, infection etc, determine the relative share of indirect (endochondral ossification) and direct (appositional) bone formation. The bone tissue initially deposited during repair of a fracture, and during skeletal development, is in an immature form characterised by a random (woven) organisation of its collagen. This bone is designated as woven bone and under the influence of functional stresses, systemic and local factors is gradually remodelled restoring the highly organised lamellar structure of mature bone (lamellar bone is composed of successive layers each of which has a highly organised infrastructure). This replacement is accomplished by resorption of old bone by osteoclasts, which adhered to bone and remove it by acidification and proteolytic
digestion, and the subsequent formation of new bone by osteoblasts\textsuperscript{60-61}. These two processes are tightly coupled to each other and are responsible for the renewal of the skeleton while maintaining its anatomic and structural integrity\textsuperscript{61}. In older persons, the rate of bone resorptions exceeds that of bone formation resulting in the condition known as osteoporosis.

The initial tissue response to an implant material is difficult to distinguish from the inflammatory response caused by the unavoidable surgical trauma. A biomaterial represents an unmovable foreign body that may not interfere significantly in the normal wound healing or may act as an injurious agent provoking a persistent stimulus to inflammation, due, perhaps, to mechanical factors, the release of degradation products, a related delayed hypersensitivity reaction or to the presence of an implant site infection\textsuperscript{1,4}. Infection (contamination of the body or part of the body by pathogenic agents, such as fungi, bacteria, viruses etc., or by the toxins that these agents may produce) associated with biomaterials represents a serious complication since the implant serves as a barrier to revascularisation of the damaged tissue immediately adjacent to it difficulting infection treatment; degradation products may inhibit macrophage chemotaxis and phagocytosis; and also bacterial colonies may reside in the glycoprotein-based film that forms on all foreign materials placed in vivo reducing their antibiotic sensitivity by 10 to 100 times\textsuperscript{4}. When the latter implant pattern behaviour occurs, the inflammatory-reparative process becomes considerably more complex, with prolonged or chronic inflammation and excessive fibroblastic repair\textsuperscript{1,62}. Macrophages remain for long periods or chronically in the vicinity of the implant\textsuperscript{4} and sometimes they develop into multinucleated giant cells, called foreign body giant cells, that often
established intimate contact with the foreign material\textsuperscript{38}. Excessive inflammation and granulation tissue formation appear to inhibit osteogenesis\textsuperscript{63} and usually, bone resorption (it seems that macrophages can attack bone directly and that, in addition, they excrete an osteoclast-activating factor promoting osteolysis\textsuperscript{4,64}), pain, swelling and redness become clinical problems giving rise to implant removal\textsuperscript{65}.

Biochemical factors may be exacerbated by biomechanical factors at the implant-bone interface since it is impossible for most biomaterials, in use today, to produce similar biomechanically stable gradients of stiffness between an implant and its host tissues as between the two types of natural bone\textsuperscript{51,66}. Ageing magnifies the problem. There are large differences between the elastic moduli of bone and those currently used orthopaedic materials (the mismatch in stiffness between bone and metallic implants is 10 to 20X depending upon location and quality of bone, and the mismatch of elastic moduli with cancellous bone is more than 100X\textsuperscript{51}). The high modulus implant will carry most of the load being the bone stress shielded which leads to its resorption and weakening\textsuperscript{51,66-69} (bone must be under some tensile load to remain healthy\textsuperscript{67}). Loosening and/or fracture of the bone, the interface, or of the implant can result\textsuperscript{51}. Debris, resulting from corrosion at the interface, will accelerate the structure weakening of the stress shielded bone due to changes of microcirculation and increased cellular activity involved in removing the foreign material\textsuperscript{51,67,70-71}.

Corrosion of orthopaedic alloys results in gradual release of soluble and insoluble compounds which differ in size, shape and composition from the bulk implant. Local host response to these foreign bodies may be, beside inflammation, elimination of the
substances with no further significant reaction (compounds could bind to proteins and be distributed throughout the body; bind to or be internalised by cells and remain localised at the wear site or become disseminated with the travel of the cell; or the ion could be oxidised and become inert) or, as suggested by several studies\textsuperscript{72-79}, proliferative responses resulting in masses of active or altered tissue with tumorigenic potential\textsuperscript{5}. Few scattered cases of implant site tumours in human orthopaedic patients were reported in the literature\textsuperscript{72-79,78-79} and most of them were associated with stainless steel fracture fixation devices and cobalt base implants\textsuperscript{4}. Although often direct causality cannot be established between implant materials and neoplastic transformation, these findings must be regarded with concern, particularly nowadays, with introduction of higher-surface-area implants into younger, more active patients which increases risk through longer expected exposure time and higher metal ion concentrations\textsuperscript{4}.

A definition of biomaterials based upon the host response was suggested by Osborn et al.\textsuperscript{80} who classified them as biotolerant, bioinert and bioactive conveying the sense of, respectively, a negative but tolerable local host response (an encapsulation by fibrous tissue of the implant is often seen; examples of these materials are stainless steel and Co-Cr alloys\textsuperscript{81}), an absence of local host response (the surrounding tissues are in close contact with the implant without fibrous encapsulation; Ti is an example of an inert material\textsuperscript{82-83}, although metallosis has been described\textsuperscript{84}) and a positive local host response (a bond is created by the material with the surrounding tissue; bioactive materials suitable for bone replacement have free calcium ions and phosphate groups at their surfaces, thus promoting a firm connection with surrounding bone; examples are calcium phosphates, hydroxylapatite and bioactive glasses\textsuperscript{85-88}).
3.2- Systemic and remote host response

Analysis of tissues, blood and urine from patients with metallic implants has revealed dissemination of degradation products throughout the body by diffusion or selective transport processes\(^5,13,89-99\). Normal body function strongly depends on precise trace-element homeostasis\(^100\) and the burden caused by "internally administrated" metal corrosion products may upset the overall balance established by physiologic levels with consequent toxicity\(^101\) (all the major alloying elements of stainless steels and Co-Cr alloys are essential in the human diet\(^1\)). Possible systemic features include metabolic (there probably does not exist a single enzyme-catalysed reaction in which either substrate, product, enzyme or some combination within this triad is not influenced in a very direct and highly specific manner by the precise nature of the inorganic ions which surround or modify it\(^100,102\), immunologic (metallic compounds may suppress immunity either by directly affecting the lymphocyte or neutrophil function or by altering the reticuloendothelial system as a whole\(^100,103-105\), eczema, urticaria, and bronchospasm have been reported in orthopaedic patients with metal hypersensitivity\(^4,100,104\)) and carcinogenic (corrosion products storage in some remote tissue depots such as liver, kidney, spleen, pancreas, lungs and heart may promote disease, and as an extreme consequence, neoplastic transformation\(^1,4,100,105\) effects\(^93,95,100-101,106-108\). The evidence for human remote site effects of implanted biomaterials is slight at this time\(^4\). However, and accordingly with the point made by Schwarz\(^109\) in 1977:

"Below a certain threshold of concentration, the organism can maintain an equilibrium. However, once the threshold level is reached, small increases in doses lead to great increases of toxic effects.... Indeed, this relationship pertains not only to all
metals but anything. It is universal, with the possible exception of mutagenicity and
carcinogenicity, but even there, repair mechanisms are at work which may give a small
area of tolerance",

there is a real and urgent need to know what are all the risk-benefit parameters for
the patient.

**BIOCOMPATIBILITY EVALUATION**

*Cell cultures*

Knowledge and characterisation of interactions of implant biomaterials with
physiological tissues, *i.e.* knowledge of the implant biocompatibility, is imperative
before materials are certified for use in human body\textsuperscript{10-11}. This assumes a particularly
important feature when one bears in mind that the majority of our existing biomaterials,
although demonstrating generally satisfactory clinical performance, were developed
based upon trial-and-error optimisation approach rather then being engineered to
produce the desired interfacial reaction\textsuperscript{2,43,62}. During the past thirty years
biocompatibility studies have been principally carried out *in vivo* on animals. However,
the length of experimentation (*ca.* 2 years), the number of animals necessary in
obtaining a certain degree of reliability and a statistical approach of the results, the legal
and ethical striving to restrict animal experimentation to a minimum\textsuperscript{115-116}, the all
important individual reactions between one animal and another, the high cost price\textsuperscript{112-114}
and the recent advances in cell and tissue culture techniques lead to the application of *in*
vitro tests to the biomaterials field. Actually, as stated by the main international and national standard agencies (International Organisation for Standardisation; American Society for Testing and Materials, Health Industry Manufacturers Association, American Dental Association, Food and Drug Administration in the USA, British Standards Institution in the UK, Association Française de Normalization in France, Deutsches Institut fur Normung in Germany, etc.), any program of assessment on biomaterials must include biocompatibility tests on cell cultures, before subsequent in vivo testing\textsuperscript{117-121}. The in vitro screening tests may be used for the following purposes\textsuperscript{117}:

(a) as screening of the candidate biomaterials for acute toxicity;

(b) on new or in-use materials to assess the release of leachables which could result in toxic effects;

(c) in the quality control performed during fabrication and sterilisation processes and on the final product.

Toxicity in vitro is a negative or deleterious effect of an agent on normal cellular biochemical functions, \textit{i.e.} a disturbance of cellular homeostasis\textsuperscript{122}. This may assume a spectrum of changes from frank loss of cellular viability to very subtle alterations in cellular function, which can be detected only by biochemical means\textsuperscript{122}. The study of the biocompatibility of medical devices involves two evaluation phases. The cytotoxicity or basal cytocompatibility\textsuperscript{112-114} testing represents the first stage and its purpose is to act as a reliable, convenient, and reproducible screening method to detect at an early stage in the
testing process cell death or other serious negative effects on cellular functions. According to the document ISO 10993-5, this can be achieved by performing tests on extracts (to detect leachable products which could exert a toxic effect), tests by direct contact (usually a cell suspension is directly seeded onto the material), and tests by indirect contact (cells are separated from the material by a diffusion barrier which prevents cells physical damage by the material). Morphology, proliferation, viability, membrane integrity and degeneration or lysis of cells are assessed in this first step and, materials showing low or no cytotoxic effects are then subject to further in vitro studies using the cell type relevant to the purpose for which the potential biomaterial has been made. In these cyocompatibility or specific cyocompatibility assays, the use of primary cells with which the material surface will come in contact once implanted in situ is recommended, wherever possible, in order to investigate the typical reactions and some particular activities of the peri-implant cells, for example, the immunomodulating activity of lymphocytes/macrophages/granulocytes, the modulation of coagulation by endothelial cells, the bone-forming activity of osteoblasts or the cyoadhesive capability of corneal cells.

For investigating the interactions between bone and artificial substrata, the osteogenic cultures should form mineralised extracellular matrix similar to that of bone tissue. The provision of the appropriate environment, nutrients, hormones, and substrate is fundamental to the expression of specialised functions and, Maniatopoulos et al. showed that culture medium has to be supplemented with dexamethasone, ascorbic acid and β-glycerophosphate to promote mineralisation in rat bone marrow cell cultures.
Dexamethasone stimulates the differentiation of progenitor cells into osteoblasts; ascorbic acid is involved in collagen synthesis and β-glycerophosphate constitutes a phosphate source for the mineralisation process. Osteoblast-like cell cultures have been obtained by the application of various techniques, from sequential collagenase digestion to mechanical isolation on different tissues (foetal and neonatal calvaria, mandibular and alveolar bone, human skeletal tissues, bone marrow, etc.) from several species including chick, rodent, rabbit, mouse, rat and human. Characterisation of these cultures is usually based on the production of certain substances associated with this type of cell, such as type I collagen, alkaline phosphatase, osteocalcin, osteonectin, bone proteoglycans, on the response to stimulation by parathyroid hormone or prostaglandin E₂, and finally on the osteogenic capacity. These cultures are ideal systems for evaluating the interactions of biomaterials and their degradation products with bone cells at the tissue-implant interface. However, in examining tissue responses to orthopaedic materials, the first in vitro experiments were performed with cells of various origins such as fibroblasts, chondrocytes, and epithelial cells and only later on, research groups have begun to use osteoblasts. Nevertheless, these studies were mainly based on morphological observations and besides physico-chemical characterisation, useful (biochemical and analytical) quantifications were not performed. In the early 1990s, several groups reported determinations of relevant histological and biochemical parameters obtained from bone cells cultured on the materials investigated (316L stainless steel, Ti-6Al-4V, Co-Cr-Mo, bioactive glass, polymethyl methacrylate, hydroxyapatite, borosilicate glass coverslips and tissue culture polystyrene) or in the presence of the materials degradation products.
Relatively little research has been done concerning the effects of orthopaedic implants corrosion products on the proliferation, metabolism and differentiation of osteoblast-like cells. Rani et al.\textsuperscript{171} reported the transformation of non-tumorigenic osteoblast-like human osteosarcoma cells to the tumorigenic phenotype by nickel sulfate. Puleo et al. conducted\textsuperscript{172-173} toxicity studies exerted by metal ions, at grossly toxic doses and subthreshold concentrations, on osteogenic cells derived from rat bone marrow stromal cells. Solutions of chromium, cobalt, iron, molybdenum and nickel ions were found to be grossly or moderately toxic to bone marrow derived cells with bone-forming potential; aluminium, manganese, titanium, and vanadium were not toxic, except at high concentrations (≥ 25 mg/L)\textsuperscript{172-173}. Other cytocompatibility studies\textsuperscript{174-175} showed that Co-Cr corrosion products and corresponding separate metal ions impair bone formation and that gene expression is not always affected in a similar way as osteoblasts. Finally, studies to assess the uptake of metal ions or calcium and phosphorus (which are involved in the mineralisation process) by osteoblasts or other studies to attempt to correlate these consumptions with cell metabolic disruption have not yet been reported in the literature.

Obviously, clear differences exist between \textit{in vitro} and \textit{in vivo} measurements. Former studies provide information only at the cellular or molecular level, with cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment\textsuperscript{128}. Their correlation with \textit{in vivo} data derived from histological, microbiological and blood analyses allow to obtain a quite good picture of the biological performance of the material\textsuperscript{117}. Furthermore, the \textit{in vitro} approach is the only possibility of testing human cells\textsuperscript{117}. However, it should be
remarked that due to the relative stagnancy of the culture medium, when compared to
the turnover of the extracellular fluid tissues, overestimation of material toxicity may
occur and, that the finite life-span of the cultured cells limits the toxicological time-
effects studies\textsuperscript{117,122}.

**Analytical techniques for quantification of metal ions released from
orthopaedic implants**

Monitoring of metal ions in biological media, such as kidney, liver, muscle, hair,
saliva, blood, serum, urine, culture medium, \textit{etc.}, obtained from retrieval implant
surgeries, or from \textit{in vivo} or \textit{in vitro} experimental studies, aid to assess the physiological
local and systemic consequences of the metallic biomaterials corrosion.

Criteria used in quantification technique selection must include sensitivity,
specificity, accuracy and precision, single \textit{vs.} multi-element capability, time of analysis,
and general applicability to biological problems\textsuperscript{176} to compensate factors such as limit
on sample availability and the large number of elements that has to be determined from
one sample, a common situation in biological analyses. Application of electrochemical
methods in the study of biomaterials is restricted essentially at ranking materials in
terms of their relative corrosion resistance\textsuperscript{43} although it is recognised that
electroanalytical methods in general and voltammetry in particular (an other important
and popular electroanalytical method is potentiometry which usually, however, do not
have limits of detection much below the micromolar level\textsuperscript{177}) provide very sensitive
routes for the quantification of many trace metals\textsuperscript{178} and biological compounds\textsuperscript{179-180}. Voltammetry comprises a group of techniques, distinguished by the type of applied potential waveform, with different concentration ranges being appropriate for the determination of high, as well as extremely low, concentrations (Fig. 3)\textsuperscript{181}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure3.png}
\caption{Overview of the different voltammetric techniques\textsuperscript{181}.}
\end{figure}

The remarkable sensitivity of stripping voltammetry is attributed to a preconcentration step in which the analyte is accumulated at the working electrode, in the majority of the cases a mercury electrode, by either faradaic (anodic or cathodic deposition) or non faradaic (adsorptive deposition; no charge is transferred during the preconcentration step) processes\textsuperscript{177}. Table 4\textsuperscript{182} represents schematically the different stripping voltammetric methods. The adsorptive approach of stripping voltammetry was
recently developed in order to offer highly selective schemes for quantifications of metals with low solubility in mercury or extreme redox potentials, and analysis of organics that, unlike metals, mostly cannot be electrodeposited\textsuperscript{183}. The adsorption of the analyte itself is, however, not the only way of accumulation in adsorptive stripping voltammetry\textsuperscript{184}. Other two ways, which are utilised for the determinations of metal ions, are represented by the reaction of the metal ion to be determined with a suitable reagent leading to the formation of a complex which is adsorbed on the surface of the electrode, or the reaction of the metal ion with the reagent adsorbed on the electrode surface\textsuperscript{184}. When coupling the adsorption accumulation with catalytic reactions, extremely low detection limits (10\textsuperscript{-12} mol/L) can be attained\textsuperscript{189-190}. Following preconcentration, the subsequent step involves the dissolution (stripping) of the deposit generating an anodic (if the analyte is oxidised) or cathodic (if the analyte is reduced) analytical signal which termed the electrolytic and adsorptive stripping techniques as, respectively, anodic or cathodic (in some cases of electroinactive compounds, only a tensammetric peak can be observed in adsorptive stripping voltammetry\textsuperscript{182,188,191}). Adsorptive stripping voltammetry is sometimes referred as adsorptive cathodic stripping voltammetry as a consequence of its usual electrochemical direction in elemental analysis\textsuperscript{177}. Each electrochemical species strips at a characteristic potential\textsuperscript{192-194}. 
### Table 4: Stripping voltammetric methods\textsuperscript{182}.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anodic Stripping Voltammetry (ASV)</td>
<td>M\textsuperscript{m+} (aq) + Hg + ne\textsuperscript{-} $\leftrightarrow$ M (Hg)</td>
</tr>
<tr>
<td>Cathodic Stripping Voltammetry (CSV)</td>
<td>2Cl\textsuperscript{-} + 2Hg $\leftrightarrow$ Hg\textsubscript{2}Cl\textsubscript{2} (s) + 2e\textsuperscript{-}</td>
</tr>
<tr>
<td>Adsorptive Stripping Voltammetry (AdSV)</td>
<td>Analyte (solution) $\leftrightarrow$ Analyte (adsorbed) (1)</td>
</tr>
</tbody>
</table>
|                                  | Striping (1) $\{\text{Reduction (AdCSV) }$
|                                  | Oxidation (AdASV) $\text{ Tensammetric dessorption}\}$ |

Among its spectrometric competitors only electrothermal atomic absorption spectrometry has nearly the same sensitivity, however at much higher costs\textsuperscript{178}. At even higher instrumentation cost, inductively coupled plasma atomic emission spectrometry offers detection limits at the parts per billion concentration level coupled with simultaneous determination capability\textsuperscript{178}. Neutron activation analysis has an overall elemental coverage with low detection limit, but at high cost\textsuperscript{178}. Associated with its high sensitivity, low cost, relative broad scope, and ability to measure simultaneously several elements, stripping voltammetry offers the possibility of studying metal speciation (oxidation state, metal-ligand interaction\textsuperscript{188}). Comparative studies with other analytical techniques have emphasised the various advantages of stripping analysis\textsuperscript{178,192-195}. The
main limitation of stripping analysis is its restriction to about 30 metals, determined by its various versions, (Fig.4)\textsuperscript{196}, it therefore does not apply to a wide range as, for example, electrothermal absorption spectrometry\textsuperscript{183}.

Figure 4- Elements measured by conventional anodic stripping voltammetry \( \square \), adsorptive stripping schemes with reduction of the element in the complex \( \square \), the ligand \( \square \), or catalytic process \( \square \)\textsuperscript{196}.

From an analytical point of view, the choice of the nature of the working electrode, solid (e.g. platinum, gold, silver, carbon) or liquid (mercury), depends on the electrochemical properties of the analyte, and the range of potentials to be explored\textsuperscript{197}. Generally, the positive potential limitations are caused by the large currents that develop due to the oxidation of water to give molecular oxygen and the negative limits arise from the reduction of water to give hydrogen\textsuperscript{198}. Electrochemical detection at positive
potentials is usually done with solid electrodes\textsuperscript{197}. Mercury electrodes, either in the form of hanging mercury drop electrode or mercury-film electrode, are the most frequently used in stripping voltammetry due to their wide cathodic potential range (until ca. -2.0 V \textit{vs.} SCE depending upon the composition of the solution in which the electrode is immersed\textsuperscript{199}), owed to the high overvoltage of hydrogen on mercury, and renewable surface\textsuperscript{200}. The application of mercury-film electrodes to stripping voltammetry was introduced by Florence\textsuperscript{201} and it combines the advantages of the conventional mercury electrode with the stability and lower charging current of solid electrode\textsuperscript{183} (the mercury film electrode has an higher surface-to-volume ratio than the hanging mercury drop electrode and consequently offers a more efficient preconcentration and higher sensitivity\textsuperscript{188}). Development of mercury-film microelectrodes (a microelectrode is an electrode with at least one dimension small enough that its properties, \textit{e.g.} mass transport regime, are a function of size; in practice the critical dimension will generally fall in the range 0.1 to 50 µm\textsuperscript{202-203}) extended these favourable properties to the microelectrode domain opening new, more effective ways of improving performance of stripping analysis\textsuperscript{197,204-205}. Mercury-film microelectrodes have been prepared in several ways using a variety of substrates\textsuperscript{206-213}. Regarding the nature of the substrates, there is no ideal support for mercury\textsuperscript{212}. Carbon is not wetted by mercury\textsuperscript{211,214} and consequently mercury "films" formed on glassy carbon or carbon fibbers are actually loosely-adhering "mercury-droplet films"\textsuperscript{213}. Improvements in films reproducibility can be achieved by using noble metals (\textit{e.g.} gold, platinum, iridium) since most of them are wettable by mercury and uniform and stable films are obtained\textsuperscript{214}. However, a disadvantage of this approach is that noble metals are solubilised in mercury (0.14% at 25°C for gold\textsuperscript{212,215}) and formation of intermetallic compounds with analyte metals deposited into the film
may occur\textsuperscript{211}. More recently, iridium\textsuperscript{204,211,213,216} has been proposed as a viable substrate for mercury deposition due to its low solubility as compared to gold and platinum (well below 10^{-6}\% by weight)\textsuperscript{215-217} however, iridium wire of diameter less than 127 μm diameter has not been commercially available difficulting the preparation of the microelectrode in micron or submicron range\textsuperscript{211}.

Microelectrodes exhibit several attractive and important properties\textsuperscript{202-203,205,218-223}. The main advantages derived from their use in stripping voltammetry are\textsuperscript{177,197,204,208,211,213,220,224-226}:

(a) the ohmic drops are greatly diminished hence measurements can be carried out even in poorly conductive media without addition of any supporting electrolyte\textsuperscript{225,227-228}; sample treatment is minimised, and \textit{in situ} analysis of fresh waters, for example, becomes, in principle, possible\textsuperscript{210}. Substantial improvement in the reliability of speciation studies in aquatic systems of low ionic strength has already been demonstrated\textsuperscript{225};

(b) the capacitive charging currents, the limiting factor of the sensitivity which can be reached in all the transient electrochemical techniques, are greatly reduced allowing a better discrimination of the faradaic current\textsuperscript{220};

(c) reduced values of resistance and capacitance of microelectrodes lower the cell-time constant, and thus allow the working electrode to assure the applied potential at a
very short time scale\textsuperscript{229} which is particularly useful for kinetics and \textit{in vivo} studies\textsuperscript{188,230-234},

(d) the enhanced mass transfer reduces the preconcentration times and eliminates the need for convective by hydrodynamics during the deposition step (these factors are particularly advantageous for analysis of biological fluids since longer accumulation times and stirring of the solution enhance the diffusion of interfering large compounds which normally diffuse very slowly to the microelectrode surface in quiescent solutions)\textsuperscript{197} increasing speed and sensitivity, and also simplifying operation and instrumentation\textsuperscript{197,202,224},

(e) another relevant aspect is the small size of microelectrodes that enables analysis of very small (microliter) sample volumes, and their use as sensors in biological systems of easily oxidised chemical substances\textsuperscript{225,231-235}.

Recently, important biomedical and environmental analytical applications have been reported relating the use of microelectrodes coupled with different chromatographic techniques or flow-injection analysis\textsuperscript{197,235-238}. However, the main problem of microelectrodes is the useful lifetime, which may be gradually lowered when real samples are analysed by passivation of the tip surface by interferences arising from the matrix\textsuperscript{180,195,234,239-240}.

An entirely different type of electrode that has given increasing attention for elemental analysis by stripping voltammetry is the chemically modified (macro or
micro) electrode\cite{177,240}. It is especially important for the analysis of metals that are incompatible with mercury\cite{177}. In chemically modified electrode, the analyte is entrapped at the electrode surface by chemical means involving a modifying agent incorporated in a polymer coating attached to the electrode surface\cite{177,180,184,240-241}.

As applied to the measurement itself, a technique that is gaining popularity in stripping analysis is square-wave voltammetry\cite{177}. The square-wave potential-time waveform\cite{242} applied to the working electrode involves the superposition of a small square-wave potential amplitude on a staircase ramp\cite{178,243-245}. The current is sampled twice during each square-wave cycle, once at the end of the forward pulse and once at the end of the reverse pulse, \textit{i.e.} only when most of the capacitive current has decayed\cite{187} (the capacitive current decreases exponentially with time, \textit{i.e.}, much faster than the faradaic current which decreases as a $t^{1/2}$ function of time\cite{181} for normal size electrodes and much less at microelectrode\cite{221}). The difference between the two measurements is plotted \textit{vs.} the base staircase potential\cite{187}. Such potential step method offers discrimination against the charging current background contribution\cite{178,183,187,193} and is even more successful when microelectrodes are used\cite{221}. The application of square-wave voltammetry with microelectrodes presented very special features\cite{226} namely, interference by dissolved oxygen when high frequencies are used is eliminated as also is, the need for stirring during the deposition step (due to the high mass transport rate as mentioned previously)\cite{211,213}. Also, this technique is especially useful for adsorptive stripping voltammetry since species are present in a surface monolayer and does not require diffusion from the electrode interior allowing the use of high sweep rates and consequently, reducing drastically the analysis time\cite{177,246-247}. The square-wave
differential peak current is proportional to the concentration of the electroactive species envolved\textsuperscript{181} and the peak potential corresponds to the half-wave potential, irrespective of the geometry of the electrode\textsuperscript{222,248}.

Like the majority of analytical techniques, stripping analysis is subject to interferences that may affect the accuracy and precision of the measurements\textsuperscript{178}. The most widely encountered are the overlap of stripping peaks of different elements present in the sample caused by a similarity in the stripping potential\textsuperscript{178,188}, the formation of intermetallic compounds which affects the peak size and position\textsuperscript{178,188}, and the presence of surface-active substances that affect the accumulation \textit{via} a competitive coverage of the electrode surface resulting in depletion of the analyte stripping peak\textsuperscript{178,182,184,188} (not all surfactants exhibit a depression effect and some may significantly enhanced the stripping peak\textsuperscript{178}). Addition of a suitable masking agent, or adjustment of the preconcentration potential or time, or the solution conditions (pH, ligand concentration, \textit{etc.}) and the use of the standard additions method are often sufficient to correct for these effects\textsuperscript{183}. Samples "rich" in interfering substances usually require a separatory technique to isolate the analyte (\textit{e.g.} ultrafiltration, chromatography, extraction procedures, \textit{etc.}\textsuperscript{184,249-250}, or \textit{in situ} separation \textit{i.e.}, at the electrode surface, by using a perm-selective polymeric coating such as cellulose acetate and Nafion\textsuperscript{251-254}) or, destruction of complexing agents or/and surface-active compounds (various surfactants are also complexing agents) by irradiation with UV light\textsuperscript{255-257} or pressurised digestion (particularly important for clinical analysis since many proteins have surface-active characteristics and complexation capacity as a result of their numerous amino, carboxyl, and sulphydryl groups)\textsuperscript{178,258-260}. 
Stripping voltammetry has proved useful for determination of numerous trace metals in environmental\(^{261-267}\), industrial\(^{268-269}\), and clinical samples\(^{258-259,270-274}\), as well as for assays of food-stuffs\(^{275-276}\), beverages\(^{277-278}\), gunshots residues\(^{279}\), and pharmaceutical formulations\(^{188,280-282}\). Its high sensitivity coupled with its non-destructive character which permits convenient cross-check analysis by independent analytical methods are obviously its greatest advantages\(^{178,186}\). In the biomedical/biomaterials field, electrothermal atomic absorption spectrometry is still, by far, the most popular analytical technique for metal ions quantification in biological tissues and fluids.

**Analytical techniques for in vitro mineralisation characterisation**

As mentioned previously, osteoblast cultures allow the performance of biocompatibility evaluations on materials to be used in orthopaedic surgery\(^{112-114,121}\). It is the function of osteoblasts to synthesise the organic components of bone and to contribute to the events resulting in its mineralisation\(^{58}\). The presence of an orthopaedic implant may interfere in the normal osteoblast phenotype development (proliferation, extracellular matrix development and maturation, and mineralisation\(^{58}\)). Informations obtained by biochemical quantification of the markers of bone cell differentiation must be complemented with data concerning bone tissue mineralisation to assess the *in vitro* material biocompatibility. Thus, monitoring of the two species involved in the mineralisation process, *i.e.*, calcium and phosphate can help to clarify the complex controls exerted by cellular activities, pH, stress, etc., and the influence of corrosion products on the biologically-induced hydroxyapatite formation and deposition\(^{283}\).
The ion-selective electrode for calcium (an ion-selective electrode is an
electrochemical sensor based on thin films or selective membranes as recognition
elements, and is an electrochemical half-cell which must be used in conjunction with a
reference electrode to form a complete electrochemical cell\textsuperscript{284}, its response is treated in
terms of the Nernst and Nikolskii equations and membrane potentials\textsuperscript{285}) has been in use
for about twenty years\textsuperscript{180,240,286-290}, and despite initial problems with sensitivity and
specificity, today improved calcium electrodes based on organic phosphates\textsuperscript{289,291-292}
(e.g. calcium bis-di-[4-(1,1,3,3-tetramethylbutyl)phenyl]phosphate\textsuperscript{291}) and on neutral
carriers\textsuperscript{240,290,293-297} (uncharged compounds that contained cavities of molecule sized
dimensions and serve as reversible and reusable binding reagents that selectively extract
the analyte into the membrane; e.g. N,N'-di-[(11-ethoxycarbonyl)undecyl]-N,N',4,5-
tetramethyl-3,6-dioxaoctane-diamide\textsuperscript{293}) are reliable and sufficiently precise and
accurate for routine analysis\textsuperscript{240,287,289}. Calcium has been determined in molten Al\textsuperscript{298},
waters\textsuperscript{299-300}, soils\textsuperscript{301}, rocks\textsuperscript{302-303}, fruits\textsuperscript{304-305}, beer and wine\textsuperscript{306}, human milk\textsuperscript{307},
saliva\textsuperscript{297,308}, urine\textsuperscript{309}, pancreatic juices\textsuperscript{310}, bile\textsuperscript{311}, cerebrospinal fluid\textsuperscript{312},
blood\textsuperscript{289-290,313-314}, plasma\textsuperscript{290}, serum\textsuperscript{296}, cell processes\textsuperscript{315-316}, etc. However, it is in fields
such as physiology, biology and medicine\textsuperscript{317,240} that the advantages of direct
potentiometric measurements or potentiometric titrations with ion-selective electrodes
(ability to selectively and non-destructively monitor the activity of a particular ionic
species in solution over a wide linear range; also, the method is not affected by colour or
turbidity, is very inexpensive, easily automated and little or no sample preparation is
required\textsuperscript{188,290,317-318}) are most convincingly demonstrated, especially in view of the
complexity of the sample media\textsuperscript{317}. The most common analyses are made in blood,
serum and urine\textsuperscript{240,288,290} and due to the composition similarity between cell culture
media and biological fluids, general analytical applicability of such electrodes to biocompatibility studies may presented the same advantages constituting, also, a valuable tool. Numerous books and reviews dealing with clinical ionised calcium determinations by means of ion-selective electrodes have been published\textsuperscript{319-324} and although the activity of the ionised calcium is claimed to be physiologically more relevant\textsuperscript{289,325-326}, assessment of total calcium concentration (\textit{e.g.} the sum of the concentrations of the ionised calcium and the calcium bound to proteins and various other complexing agents) is also an useful parameter particularly\textsuperscript{296,327-330} in mineralisation studies\textsuperscript{121,331-332}.

Detection by means of ion-selective electrodes covers an exceedingly broad concentration range, which, for certain electrodes, may embrace five orders of magnitude\textsuperscript{188}. In practice, the usable range depends on other ions in the solution\textsuperscript{188} and by no means, is the selectivity high enough with all electrodes that it is possible to make a direct, interference-free measurement of the desired ion in the presence of all manner of other ions\textsuperscript{317}. Often the composition of the test solution must be known to some degree in order to assess suitable preventive measures (complexation, precipitation or ion-exchange), so that interferences from other ions also sensed by the electrode can be reduced or avoided\textsuperscript{317}. Summarising, evaluation of the sensor response (\textit{i.e.}, sensitivity, selectivity, stability and response time) every time it is placed in a new matrix is therefore necessary.

Alteration of phosphate concentration in cell culture medium throughout the incubation time of the \textit{in vitro} biocompatibility tests\textsuperscript{138,333} may be determined suitably by
spectrophotometric assays based on the formation of a coloured complex\textsuperscript{334-338}. This evaluation is particularly important since alkaline phosphatase, considered an osteoblast phenotype marker\textsuperscript{57,150}, is suspected to be involved in the mineralisation process by hydrolysing organic phosphates to release inorganic phosphate at sites of mineralisation and, together with its calcium binding properties, regulating and ensuring the presence of both ions in sufficient concentrations at localised sites to initiate mineralisation\textsuperscript{138,333}.

Combined with the chemical analyses of the supernatant fluid, observation, by light and scanning electron microscopy, and analysis of the mineral in the cultures by histochemical staining\textsuperscript{339} for calcium and phosphates, Fourier Transform infrared spectroscopy\textsuperscript{333,339}, energy dispersive X-ray microanalysis or/and X-ray diffraction\textsuperscript{339} are also advisable to characterise and compare the distribution and the nature of the mineral deposited \textit{in vitro}, in the absence or in the presence of corrosion products, with the one observed \textit{in vivo}. 
AIMS OF THE STUDY

The aims of the present work were:

(a) to develop suitable analytical procedures (i) to quantify down to the part per billion level, using microelectrodes, the major metal ions that are released during the corrosion of a 316L stainless steel (C max. 0.03, Cr 16.0-18.0, Ni 10.0-14.0, Mo 2.00-3.00, Mn max. 2.00, Si max. 1.00, P max. 0.045, S max. 0.030, Fe balance; wt \%\(^1\)) orthopaedic implant and (ii) to help to monitor the mineralisation process by measuring the changes in concentration of total and ionised calcium and phosphorus.

(b) to investigate the in vitro (specific) cytocompatibility of 316L stainless steel corrosion products and the corresponding major metal ions (iron, chromium and nickel), with particular focus on the dose- and time-effects on the rabbit, rat and human osteoblast-like cells, using the developed analytical techniques combined with biochemical, histochemical and morphological assays.

The methods and materials used, and results obtained in the studies undertaken to achieve these goals as well as discussions are presented in detail in the next section of this thesis (section II- chapters 1-5).
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II- STUDIES UNDERTAKEN
II- Chapter 1

1- Development and Characterisation of Osteoblastic Cell Cultures Used in 316L Stainless Steel Cytocompatibility Studies
1.1- *In Vitro* Biomineralisation by Osteoblast-Like Cells:  
Part I- Retardation of Tissue Mineralisation by Metal Salts

**ABSTRACT**

The cytocompatibility of 316L stainless steel (316L SS) corrosion products was investigated with particular focus on the dose- and time-effect of electrochemically dissolved 316L SS and the corresponding separate metal ions, on osteogenic bone marrow derived cells. Type AISI 316L stainless steel was anodically dissolved in Hank's Balanced Salt Solution (HBSS) and diluted to the following concentrations: 500 μg/mL of Fe, 122 μg/mL of Cr and 101 μg/mL of Ni, as estimated by atomic absorption spectrometry. Similarly, salt solutions containing 500 μg/mL of Fe (FeCl₃·6H₂O), 122 μg/mL of Cr (CrCl₃·6H₂O) or 101 μg/mL of Ni (Ni(NO₃)₂) were prepared. All solutions were diluted 1:10³, 1:10⁴ and 1:10⁵ and their effects on cell proliferation and function of rabbit bone marrow cells were studied up to 28 days of culture. Bone marrow cells (second subculture) were cultured in α-Minimal Essential Medium (α-MEM) supplemented with 10% foetal bovine serum, 10⁻⁸ mol L⁻¹ dexamethasone, 2.52x10⁻⁴ mol L⁻¹ ascorbic acid and 10⁻² mol L⁻¹ β-glycerophosphate. The osteoblast response to the presence of metal ions was evaluated by biochemical assays (enzymatic reduction of MTT for evaluation of cell viability/proliferation, and estimation of alkaline phosphatase (ALP) activity) and histochemical assays (identification of ALP positive cells and calcium and phosphates deposits). Results suggest a decrease in the expression of the osteoblast phenotype in the presence of ion and alloy solutions. Stainless steel corrosion products elicited slight effects but the corresponding metal ions produced pronounced effects on the osteoblast phenotype, namely, an alteration in the levels and temporal expression of ALP and lower and retarded tissue mineralisation ability.
INTRODUCTION

The cellular events taking place at the bone/material interface can be determinant for the success of the implant long term performance. Ion and particles release from metal implants remains a concern because of their potential role in various pathological bone and tissue conditions\textsuperscript{1-5}. Knowledge of the toxic effects of ions on cells and tissues will be of significance in understanding the phenomenon of osteolysis and loosening of orthopaedic implants. Well-characterised osteoblast-like cell cultures provide a suitable \textit{in vitro} model to study the effects that ions may have on bone cells at the tissue-implant interface, as they are ideal systems for dose-effect quantification of biomaterial degradation products\textsuperscript{6}. Previous studies performed in osteogenic cell cultures suggest acute and long-term toxic effects of metal ions found in commonly used orthopaedic implants on proliferation and function of osteoblast lineage cells\textsuperscript{6-9}. The main objective of the present work was to study the dose- and time-effect of 316L SS corrosion products and the major corresponding metal ions (Fe, Cr and Ni) on proliferation and function of osteoblast-like cells from rabbit bone marrow. Rabbit bone marrow was cultured in experimental conditions reported to favour osteoblast differentiation and results concerning enzymatic reduction of MTT (for evaluation of cell viability/proliferation), alkaline phosphatase activity (biochemical and histochemical evaluation) and ability to form mineralised tissue (by histochemical identification of calcium and phosphate deposits) found in control and treated cultures are reported.
MATERIALS AND METHODS

Metallic slurries

Type AISI 316L stainless steel (Fe 63.9%, Cr 18.0%, Ni 12.5%, Mo 2.8%, Si 1.2%, Mn 1.6%, P 0.045% and C 0.025%, weight for weight) was anodically dissolved in HBSS, which simulates the composition of physiological fluids. The resulting concentrations of the major metal ions in the 316L slurry were, after dilution and as determined by atomic absorption spectrometry (AAS): 500 μg/mL of Fe, 122 μg/mL of Cr and 101 μg/mL of Ni.

Salt solutions containing 500 μg/mL of Fe (FeCl₃.6H₂O), 122 μg/mL of Cr (CrCl₃.6H₂O) or 101 μg/mL of Ni (NiNO₃) were prepared separately in HBSS.

Cell culture

Osteogenic cells were obtained from rabbit bone marrow. Briefly, one tibia was removed and washed twice with α-MEM (α-Minimum Essential Medium, SIGMA M 0894) containing antibiotic antimycotic solution (SIGMA A 9909) 10X concentrated (1000 units of penicillin per mL, 10 mg/mL of streptomycin and 25 μg/mL of amphotericin B). Bone marrow pieces were distributed evenly onto a 14 cm diameter dish and covered with α-MEM supplemented with 10% foetal bovine serum, 10% antibiotic antimycotic solution 10X concentrated, $10^{-8}$ mol L$^{-1}$ dexamethasone,
2.52x10^{-4} \text{ mol L}^{-1} \text{ ascorbic acid and } 10^{-2} \text{ mol L}^{-1} \text{ } \beta\text{-glycerophosphate. A volume of } 20 \text{ mL of supplemented medium was added 16 hours later. Rabbit bone marrow cells were cultured at 37}^\circ\text{C, in a humidified atmosphere containing 5\% } \text{CO}_2 \text{ and 95\% air. Medium containing non-adherent cells was removed on day 4, and passages were done after trypsinisation of adherent cells in exponential growth (7 days after the beginning of the incubation).}

Cells of the second passage were grown for 7, 14, 21 and 28 days in control conditions and in the presence of stainless steel corrosion products and also the corresponding metal ions. Cells were seeded at a concentration of 10^4 \text{ cells/cm}^2: \text{ a) in 35 mm diameter tissue culture dishes for histochemical assays, b) in 24 well culture plates for alkaline phosphatase activity assay, c) and in 96 wells culture plates for cell viability MTT assay. Stainless steel corrosion products were added to some cell cultures in three concentrations, namely: } \text{SS}^- \text{ (} = 0.005 \text{ } \mu\text{g/mL Fe } + 0.001 \text{ } \mu\text{g/mL Cr } + 0.001 \text{ } \mu\text{g/mL Ni}), \text{SS}^0 \text{ (} = 0.050 \text{ } \mu\text{g/mL Fe } + 0.012 \text{ } \mu\text{g/mL Cr } + 0.010 \text{ } \mu\text{g/mL Ni}) \text{ and SS}^+ \text{ (} = 0.500 \text{ } \mu\text{g/mL Fe } + 0.122 \text{ } \mu\text{g/mL Cr } + 0.101 \text{ } \mu\text{g/mL Ni}). \text{ Similarly, iron, chromium and nickel salt solutions were added separately to other culture dishes at equivalent concentrations, } i.e.: \text{ Fe}^- \text{ (} 0.005 \text{ } \mu\text{g/mL)}, \text{ Fe}^0 \text{ (} 0.050 \text{ } \mu\text{g/mL}) \text{ and Fe}^+ \text{ (} 0.500 \text{ } \mu\text{g/mL); Cr}^- \text{ (} 0.001 \text{ } \mu\text{g/mL)}, \text{ Cr}^0 \text{ (} 0.012 \text{ } \mu\text{g/mL}) \text{ and Cr}^+ \text{ (} 0.122 \text{ } \mu\text{g/mL); Ni}^- \text{ (} 0.001 \text{ } \mu\text{g/mL)}, \text{ Ni}^0 \text{ (} 0.010 \text{ } \mu\text{g/mL}) \text{ and Ni}^+ \text{ (} 0.101 \text{ } \mu\text{g/mL}). \text{ For control experiments, normal culture medium was added to another set of cell cultures. The culture media were changed twice a week, collected and stored at -20^\circ\text{C for further analysis. Control}
and metal treated cultures were tested at days 7, 14, 21 and 28 to perform the biochemical and histochemical assays.

**Biochemical assays**

**MTT assay**

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple Formosan product) was used to estimate cell viability and proliferation. Cells (8 wells for each situation) were incubated with 0.5 mg/mL of MTT in the last 4 hours of the culture period tested. The media were then decanted, Formosan salts were dissolved with 100 μL of dimethylsulphoxide and the absorbance was determined at 600 nm in a ELISA reader.

**Alkaline phosphatase activity**

ALP was assayed based on the hydrolysis of p-nitrophenylphosphate (used as substrate) into p-nitrophenol at 37°C and pH=10.3. After removal of culture medium, the tissue was washed twice with phosphate buffered solution (PBS), detached from the culture dish with a rubber scraper after addition of PBS with Triton X-100 to each well, and sonified. Then, 100 μL of substrate (a 20 mmol L⁻¹ solution) was added to 100 μL of each sample and the mixture was incubated at 37°C during 30 min. The reaction was stopped by the addition of 500 μL of NaOH 0.2 mol L⁻¹. Optical density was determined at 405 nm on a ELISA reader and compared with the values of a series of p-nitrophenol standards.
Before performing the biochemical assays, it was observed that the metal ions present in the culture medium did not cause any interference in the MTT and ALP biochemical measurements.

**Histochemical methods**

The presence of ALP positive cells and phosphate or calcium deposits were visualised by histochemical staining. The cultures were fixed with 1.5% glutaraldehyde in 0.14 mol L\(^{-1}\) sodium cacodylate buffer and rinsed with distilled water.

**ALP staining**

Fixed cultures were incubated during 1 hour in the dark with a mixture, prepared in Tris buffer pH=10, containing 2 mg/mL of Na-\(\alpha\)-naphtyl phosphate (reacting substrate) and 2 mg/mL of fast blue RR salt (diazotate-4-benzoylamino-2,5-dimethoxyaniline, diazorium salt that binds to phosphate ions to form a brown to black precipitate). The incubation was stopped by rinsing the samples with water and then, the cells were observed in a Olympus BH-2 optical microscope. ALP positive cells stained brown.

**Phosphate staining**

Phosphate deposits were assessed by the von Kossa technique\(^{10}\) i.e., the fixed cultures were covered with a 1% silver nitrate solution and kept for 1 hour under UV light. After rinsing, a 5% sodium thiosulphate solution was added for 2 min. and cultures were washed again. Phosphate deposits stained black.
Calcium staining

The fixed cultures were covered with a 1.0% S alizarin sodium sulfonate solution (0.028% in NH₄OH), pH=6.4, for 2 min. and then rinsed with water and acid ethanol (ethanol, 0.01% HCl)¹⁰. Calcium deposits stained red.

Statistical analysis

The percent alterations of MTT reduction and ALP activity in respect to the values found in control and stainless steel treated cultures were calculated according to the formula:

\[
\text{% alteration} = \left[ \frac{\text{(value of absorbance of the tested metal ions} - \text{value of absorbance of the control)}}{\text{(value of absorbance of the control)}} \right] \times 100
\]

Data were analysed statistically using the double-sided t-test. All data presented represent the mean of, at least, three replicates (ALP) and the differences observed between groups were considered significant for \(p\) values lower than 0.05.

RESULTS AND DISCUSSION

In the present work, rabbit bone marrow was cultured in experimental conditions reported to favour osteoblast differentiation in several bone cell culture systems, namely, in the presence of ascorbic acid, β-glycerophosphate and dexamethasone⁶-⁷,¹¹-²². The second subculture was used to compare the effects of stainless steel corrosion
products and the corresponding metal ions (Fe, Cr and Ni) in the long-term behaviour of osteoblastic cells.

In control conditions, cells proliferated gradually with incubation time, reaching a stationary phase around day 21; cultures presented high levels of ALP and maximal values were observed approximately at day 14 and, after that, activity of the enzyme dropped significantly\(^7\). Formation of mineral deposits, demonstrated by the positive staining of alizarin red and von Kossa assays, occurred in the third week of culture (between days 14 and 21), following the maximal ALP activity\(^7\). ALP has long been associated with biological calcification and these observations are in agreement with results reported in the literature for other culture systems\(^11-12,15-17,19-20,22\).

**Cell viability/proliferation**

Despite some controversy, reduction of the tetrazolium salt MTT by cellular enzymes has been widely used to measure cell viability/proliferation\(^22-23\) and to estimate drugs and biomaterials toxicity\(^6-7,23-28\). Osteoblast-like cells were cultivated in control medium or exposed to SS\(^-\), SS\(^0\) and SS\(^+\) solutions, Fe\(^-\), Fe\(^0\) and Fe\(^+\) solutions, Cr\(^-\), Cr\(^0\) and Cr\(^+\) solutions, or Ni\(^-\), Ni\(^0\) and Ni\(^+\) solutions for 7 to 28 days. Fig. 1 shows results concerning cell viability/proliferation found in these cultures, expressed as percentage alteration relative to the control.

Stainless steel corrosion products used at different concentrations (SS\(^-\), SS\(^0\), SS\(^+\)) caused, in general, no significant effects \((p>0.05)\) in cell viability/proliferation, as
compared to the control culture, although, in the presence of SS⁰ and SS⁻ a trend for stimulation was observed, especially evident at day 21. In contrast, metal ions tested separately (Fe, Cr or Ni) at corresponding concentrations caused significant increase ($p<0.05$) in cell viability/proliferation (Fig. 1b-d). However, a different pattern of variation was found in the presence of the three ions. In cultures exposed to Fe ions, stimulation of cell viability/proliferation occurred in the first two weeks of culture and maximal values were observed around day 14 (Fe⁰ caused the highest MTT reduction stimulation). Cultures exposed to Cr ions showed an increase in cell proliferation during the incubation time tested, although, stimulation of MTT reduction was more significant in the first week (maximal levels were observed around day 7); stimulation was higher in the presence of Cr⁰. In the presence of Ni ions, cultures showed somewhat mixed results, peak levels at day 7 (Ni⁰), between days 14-21 (Ni⁻) and variable effects (Ni⁺). The effects of metal ions on MTT reduction appear to be more pronounced at the lower concentrations tested.
Figure 1- Percentage alteration in the MTT assay relative to the control. Effects of the three concentrations of the (a) SS corrosion products; (b) Fe; (c) Cr and (d) Ni.

*Significant differences (p<0.05) between groups of metal exposed cells and control cells.
Fig. 2 shows results concerning the percentage alteration in MTT reduction for SS<sup>0</sup>, Fe<sup>0</sup>, Cr<sup>0</sup> and Ni<sup>0</sup> relative to the control (Fig. 2a) and for Fe<sup>0</sup>, Cr<sup>0</sup> and Ni<sup>0</sup> over SS<sup>0</sup> samples (Fig. 2b). Results show that the effects of SS<sup>0</sup> and the corresponding metal ions in cell proliferation differ in terms of quantitative response (values similar to those observed in control medium in the presence of SS<sup>0</sup> solution and significantly higher in the presence of the metal ions) and also pattern of variation (trend for an increase towards the end of the culture with SS<sup>0</sup> and stimulation more evident during the first two weeks of culture with the metal ions).

![Graph](image_url)

**Figure 2**- Percentage alteration in the MTT reduction for the SS<sup>0</sup>, Fe<sup>0</sup>, Cr<sup>0</sup>, and Ni<sup>0</sup> concentrations relative to the control (a) and for Fe<sup>0</sup>, Cr<sup>0</sup>, and Ni<sup>0</sup> concentrations relative to the SS<sup>0</sup> samples (b). *Significant differences (p<0.05) between groups of metal exposed cells and control cells.
Light microscope observation of osteoblast-like cells showed that by day 7 all cultures (control and treated) were confluent (Fig.3a), and by day 14 the cell layer began to detach from the surface culture forming a mass of roll tissue (Fig.3b). From this aggregation new cells were proliferating towards the cell dish (Fig.3c). The formation of such a mass of roll tissue has been observed quite often not only in our laboratory but also elsewhere\textsuperscript{29}. These results are in agreement with Wrouwenvelder \textit{et al.}\textsuperscript{29} who have reported that “after confluence it is often observed that the formed cell layer detaches spontaneously from the underlying (non reactive) substrate” or/and since after confluence, cells grew forming multilayers and cells attached to the bottom of the plate may begin to die (and detach) due to the lack of nutrients and senescence.
Figure 3. Light microscope photographs of cell cultures showing (a) cells in confluence (X40); (b) the mass of roll tissue formed by day 14 (X40) and (c) new cells proliferating from the mass of roll tissue (X200).
Alkaline phosphatase activity

ALP activity has been routinely used in *in vitro* experiments as a relative marker of osteoblast differentiation. Osteoblast-like cells were identified on tissue culture dishes by showing a positive staining reaction for alkaline phosphatase (ALP) in histochemical assays carried out in cultures grown in the absence or in the presence of SS⁻, SS⁰ and SS⁺ solutions, Fe⁺, Fe⁰ and Fe⁺ solutions, Cr⁺, Cr⁰ and Cr⁺ solutions, or Ni⁺, Ni⁰ and Ni⁺ solutions. Similarly, to estimate the production of ALP, osteoblast-like cells were cultured in control medium or exposed to the above metallic solutions and were analysed weekly by biochemical means (see Materials and Methods). Results are shown in Figs. 4 and 5.

Fig. 4 shows results concerning dose and time-dependent effects of alloy and metal solutions on ALP activity, relative to the control. Fig. 5 compares the effects of SS⁰, Fe⁰, Cr⁰ and Ni⁰ over control culture (Fig 5a) and the effects of Fe⁰, Cr⁰ and Ni⁰ over SS⁰ treated cultures (Fig. 5b). The solutions tested produced mixed results on osteoblast-like cells alkaline phosphatase activity (Figs. 4 and 5). A common effect was a significant decrease in ALP activity during the second week of culture and, at day 14, levels of the enzyme were very low as compared to the control. It is interesting to note that in this culture system and in control conditions, ALP is most rapidly produced around day 14. Stainless steel slurry caused significant lower effects in ALP activity (Figs. 4a and 5a) than the corresponding metal ions (Figs. 4b-d and 5a), although, in the presence
Figure 4. Percentage alteration in the ALP assay relative to the control. Effects of the three concentrations of the (a) SS corrosion products; (b) Fe; (c) Cr and (d) Ni.

*Significant differences (p<0.05) between groups of metal exposed cells and control cells.
Figure 5- Percentage alteration in the ALP reduction for the SS₀, Fe₀, Cr₀, and Ni₀ concentrations relative to the control (a) and for Fe₀, Cr₀, and Ni₀ concentrations relative to the SS₀ samples (b).
*Significant differences (p<0.05) between groups of metal exposed cells and control cells.

of SS₀ and SS⁻ a trend for ALP activity inhibition was observed, with an evident decrease in the levels of the enzyme at day 14. At days 7 and 21, Fe and Cr solutions caused significant increases in ALP activity. However, as shown before, cell proliferation was also stimulated in the presence of these solutions (Fig. 1). It is interesting to note that the percentage of stimulation in MTT reduction was, in general, higher than the percentage of increase in ALP activity in the presence of these metal ions, over control cultures (Figs. 1 and 4). Ni solutions had no effect in ALP activity, despite of the significant increase in cell proliferation observed in the presence of this ion. These results suggest that metal solutions appear to stimulate proliferation of a cell
population with lower producing ALP ability than that growing in control conditions; this effect appears to be maximal in the presence of Ni solutions. SS corrosion products show a trend for a similar effect, although at a much lower extent (low stimulation in MTT reduction and low inhibition in ALP activity; Figs. 1a and 4a).

Results concerning ALP activity in normal and treated cultures do support the general conclusion that the solutions tested can affect the levels and temporal expression of ALP in rabbit osteoblast-like cell cultures.

Histochemical assays for calcium and phosphate deposits showed that the mineralisation process was retarded in all cultures exposed to metallic solutions. Intensity of the staining was clearly lower, especially in cultures treated with Cr and Ni solutions.

Studies on the osteogenic cell system support the concept that osteoprogenitor cells differentiate into pre-osteoblasts, mature osteoblasts and then into osteocytes in discrete phases\(^30-34\). ALP activity is present when cells become recognised as pre-osteoblast and osteoblast (but it is absent from the osteocyte) and it is generally accepted that as the specific activity of ALP in a population bone cells increases there is a corresponding shift to a more differentiated state\(^11,16-17,30-34\). ALP appears to play a crucial role in the initiation of matrix mineralisation and, after this, expression of the enzyme is down-regulated\(^15-16,32-34\).
In this study, in control conditions, ALP attained maximal levels around day 14 and formation of mineral deposits occurred following the maximal ALP activity (after day 14). Synthesis of ALP by osteoblast-like cells appears to decrease in the presence of the metal solutions (effect suggested by analysing the results concerning percentage of alteration in cell viability/proliferation and in ALP activity in control and treated cultures). In addition, the significant inhibition in ALP activity observed in cultures treated with the metal solutions around day 14 suggests the possibility of an impairment in the mineralisation process in these experimental situations. Decreased histochemical staining for calcium and phosphates deposits observed in metal ions treated cultures also supports this view.

Differences in effects between corrosion products from metal alloys and single metals, concerning proliferation and function of fibroblast and osteoblast-like cell cultures have been previously reported\textsuperscript{6,7,28} and should be a matter of further investigation in order to clarify the interaction of metal ions with biological systems.

**CONCLUSIONS**

At the experimental conditions described, stainless steel corrosion products and the corresponding metal ions affect proliferation and function of osteoblast-like cell cultures. Results concerning cell viability/proliferation, ALP activity and histochemical identification of mineral deposits in control and treated cultures suggest a decrease in the expression of the osteoblast phenotype in the presence of ion and alloy solutions.
Stainless steel corrosion products elicited slight effects but the corresponding metal ions (Fe, Cr and Ni) produced pronounced effects on the osteoblast phenotype, namely, an alteration in the levels and temporal expression of ALP and lower and retarded tissue mineralisation ability. Caution should be taken when comparing biological effects caused by separate metal salt solutions and those from metallic solutions derived from electrochemical dissolution of an alloy.

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II- CHAPTER 2

2- DEVELOPMENT OF ELECTROANALYTICAL PROCEDURES FOR QUANTIFICATION OF 316L STAINLESS STEEL CORROSION PRODUCTS AND CORRESPONDING MAJOR SEPARATE METAL IONS IN CELL CULTURE MEDIUM, AND ALSO FOR EVALUATION OF THE IN VITRO MINERALISATION PROCESS
2.1- Iron Determination in Osteoblast-Like Cell Culture Medium by Adsorptive Cathodic Stripping Voltammetry With a Mercury Microelectrode

ABSTRACT

Mercury film microelectrodes were used to determine iron in osteoblast-like cell culture medium by performing adsorptive cathodic stripping voltammetry in the presence of catechol. The optimised analytical conditions are a catechol concentration of $5.00 \times 10^{-4}$ mol L$^{-1}$, pH 8.0 and a collection potential of -1.80 V which eliminates the copper interference. The re-oxidation period was limited to the time between switching to -0.10 V (vs. Ag/AgCl) and scanning up to the metal peak (ca. -0.40 V). The limit of detection obtained for a 30 s collection time was $1.93 \times 10^{-8}$ mol L$^{-1}$ iron. The results achieved by this procedure and using the square-wave voltammetry technique are accurate and precise. Biological assays were performed to evaluate the mineralisation process.
INTRODUCTION

The biomedical applications of stainless steels in bone replacement and dentistry have been widely practised due to their high generalised corrosion resistance, workability conditions, good mechanical properties and relatively low price\(^1\). Despite the good corrosion resistance of AISI 316L stainless steel (the most commonly used metallic implant in orthopaedics surgery), the accumulation of metal ions at the implant site and their distribution throughout the body, via systemic circulation, have been pointed out, in the last ten years, as a cause of health concern\(^2\)-\(^{10}\). The technique conventionally used to quantify the amounts of metal ions released from the metallic implants is atomic absorption spectrometry (AAS). Nevertheless, voltammetric methods coupled with suitable digestion procedures provide a very efficient and reliable alternative\(^{11}\)-\(^{13}\), combining extreme sensitivity with high accuracy, good precision and short analysis time\(^{14}\). The application of the electrochemical approach to the field of biomaterials presents some advantages since it constitutes a non-destructive method. This fact allows to determine other important biological parameters (e.g. Ca, P) within the same sample, starting all the analysis with a small sample volume due to the high sensitivity (large dilutions can be made) that characterises stripping voltammetry.

Several authors have reported in the literature, polarographic\(^{15}\) or voltammetric procedures\(^{16}\)-\(^{18}\) to determine iron in aqueous solutions showing that low levels can be determined using adsorptive cathodic stripping voltammetry (CSV) in the presence of catechol\(^{16}\), Solochrome Violet RS\(^{17}\) and 1-nitroso-2-naphthol\(^{18}\).
The aim of this study is to investigate the biodegradation and mineralisation processes that occur at the AISI 316L stainless steel surface by performing *in vitro* tests\textsuperscript{19-21} using well-characterised osteoblasts (cells that promote bone growth)\textsuperscript{22-23}, as they are ideal systems for dose-effect quantification of biomaterials degradation products\textsuperscript{24}. Different concentrations of iron (the major component of stainless steels) were added to the cultures to evaluate the effects caused in the cell growth and mineralisation since some clinical reports\textsuperscript{25} suggested that localised tissue iron overload and iron toxicity occur around some corroded stainless steel implants. The unhealthy consequences which have been associated with a massive iron overload are: reduced bone formation, decreased quantity of bone deposited during one remodelling period, iron deposit inside the bone, impaired bone mineralisation, tissue necrosis and osteoporosis\textsuperscript{36}. The iron levels in the culture medium were quantified by adsorptive CSV using a mercury film microelectrode (MFM) and by AAS for comparison of the experimental data.

**MATERIALS AND METHODS**

**Reagents**

**Osteoblast-like cell culture reagents**

The osteoblast-like cell culture medium consisted of α-MEM (α-Minimal Essential Medium SIGMA M0894) supplemented with 10% of antibiotic antimycotic solution 10 times concentrated (solution with 1000 units of penicillin per mL, 10 mg/mL of
streptomycin and 25 μg/mL of amphotericin B), 10% of foetal bovine serum, 10^8 mol L^{-1} of dexamethasone, 10^{-2} mol L^{-1} of β-glycerophosphate and 2.52x10^{-4} mol L^{-1} of ascorbic acid. α-MEM is a buffered solution of pH 7.2 ± 0.3 containing essentially inorganic salts (calcium chloride, magnesium sulfate, potassium chloride, sodium chloride, sodium phosphate monobasic and sodium bicarbonate), 21 amino acids and 11 vitamins.

Type AISI 316L stainless steel (Fe 63.9%, Cr 18.0%, Ni 12.5%, Mo 2.8%, Si 1.2%, Mn 1.6%, P 0.045% and C 0.025% weight for weight) was anodically dissolved in Hank's Balanced Salt Solution (HBSS), which simulates the composition of the extracellular physiological fluids, by imposing a constant potential of ca. 4 V during five hours. The resulting concentrations of the major metal ions in the prepared stock slurry were determined by AAS: 8.95x10^{-3} mol L^{-1} of Fe, 2.35x10^{-3} mol L^{-1} of Cr and 1.72x10^{-3} mol L^{-1} of Ni. A salt solution of FeCl_3·6H_2O containing 8.95x10^{-3} mol L^{-1} of iron was prepared separately in HBSS.

**Voltammetric reagents**

The mercury deposition solution was a deoxygenated solution containing 1.00 mol L^{-1} KNO_3 plus 5.70x10^{-3} mol L^{-1} Hg(NO_3)_2 and 0.500% concentrated nitric acid^{27}.

A buffer solution of 0.100 mol L^{-1} PIPES (piperazine-N,N'-bis-2-ethanesulfonic acid) with a pH value of ca. 8.0 was prepared in 0.500 mol L^{-1} NH_4OH. A stock solution
of 0.100 mol L\(^{-1}\) catechol was prepared daily and oxygen was removed immediately by purging with nitrogen to prevent the oxidation of catechol to the corresponding \(\sigma\)-quinone\(^{28}\).

Standard solutions of Fe\(^{3+}\) were prepared daily by dilution of a 1.70x10\(^{-2}\) mol L\(^{-1}\) BDH atomic absorption spectrometric standard solution whereas standard solutions of Fe\(^{2+}\) were prepared by dissolving the appropriate weight of iron ammonium sulphate in deionised and triply distilled water.

All reagents used were of suprapure (mineral acid, Merck) or analytical reagent grade (Merck). Deionised and triply distilled water was used for preparing all solutions.

**Procedures**

**Sample preparation**

Osteoblast-like cells were obtained from rabbit tibia bone marrow, were seeded into multi-well culture plates and grown in osteoblast-like cell culture medium\(^{29}\). The prepared metallic physiological slurry was added to the cell cultures, except to those used as control, in several concentrations namely, 1.00x10\(^{-1}\)%, 1.00x10\(^{-2}\)% or 1.00x10\(^{-3}\)% (% relative to the stock slurry). Similarly, iron salt was added at equivalent concentrations, \(i.e.,\ 8.95\times10^{-6}\) mol L\(^{-1}\), 8.95x10\(^{-7}\) mol L\(^{-1}\) or 8.95x10\(^{-8}\) mol L\(^{-1}\). Cultures were incubated in a humidified atmosphere with 95% air and 5% CO\(_2\) at 37°C and were sacrificed at days 7th, 14th, 21st and 28th to perform the biochemical and histochemical assays. Samples of culture medium were collected and stored at -20°C.
Quantification and electrochemical studies of iron in osteoblast-like cell culture medium required mineralised samples. Digestion of samples with concentrated nitric acid (65%) was carried out in a CEM Model MDS-2000 microwave oven. After the digestion period, the decomposition solution was evaporated to fumes with perchloric acid\textsuperscript{30}. The residues were dissolved in a volume of deionised and triply distilled water to the initial volume of the sample, \textit{i.e.} the volume before destruction of the organic matter. This procedure was done either for electrochemical measurements and for AAS analyses.

\textit{Voltammetric studies}

The voltammetric studies were performed with an AUTOLAB potentiostat/galvanostat model PSTAT 10 coupled with an ECD Module (EcoChemie), a working MFM, an Ag/AgCl reference electrode and a cylindrical carbon counter electrode. The MFM was prepared by electrodeposition of a mercury film onto a gold microdisk (radius=12.5x10\textsuperscript{-6} m) by the application of a constant potential of 0.00 V\textsuperscript{27} during a deposition time of 60 s.

For the electrochemical study of iron in osteoblast-like cell culture medium, samples (after, at least, a four time dilution with deionised and triply distilled water) were pipetted into a polargraphic cell (volume capacity of 3-15 mL) and the pH was adjusted to 7.9-8.1 by addition of 0.100 mol L\textsuperscript{-1} PIPES buffer. The working MFM was inserted in the solution and then, the dissolved oxygen was removed by purging with nitrogen during 12 minutes (the large bubbles of nitrogen did not disrupt the mercury film as mentioned already by Huizenga \textit{et al.}\textsuperscript{31}). Finally, catechol was added. The final
concentration in the sample of the buffer, and of the complexing agent were 1.00x10^{-2} \text{ mol L}^{-1} \text{ and } 5.00x10^{-4} \text{ mol L}^{-1}, \text{ respectively.}

In this study, the technique of square-wave voltammetry was used. Scans were preceded by a deposition step at -1.80 V during a deposition time of 30 s and were initiated at -0.10 V. The square-wave parameters selected for iron quantification were a frequency of 50 Hz, an amplitude of 20 mV and a staircase step of 4 mV. Due to the inherent properties of microelectrodes, no forced convection during the deposition step before the cathodic scan was used.

Determination of iron in the biological samples by the technique of AAS was performed with a Zeeman Atomic Absorption Spectrometer Model 4100 ZL from Perkin Elmer and using Mg(NO_3)_2 as the matrix modifier.

**RESULTS AND DISCUSSION**

As reported previously in the literature, low levels of iron, in the nanomolar range, can be determined using a chelating agent such as catechol\textsuperscript{16}, Solochrome Violet RS\textsuperscript{17} or 1-nitroso-2-naphthol\textsuperscript{18}. The procedure developed by the van den Berg's group\textsuperscript{16} for determination of iron in sea water using catechol was selected since it enabled us to quantify total iron content (in contrast with Solochrome Violet RS) and the limit of detection was adequate. However, in order to test the effect of the biological matrix on the stripping process, a series of optimisation studies were carried out. Several operating
parameters were investigated, namely, reproducibility, deposition potential, pH and buffer concentration, catechol concentration, deposition time, the detection limit and the linearity range in samples of osteoblast-like cell culture medium.

Reproducibility

Repeated adsorptive CSV scans in culture medium digests revealed that variations in the iron peak height smaller than 3%, were only attained ca. fifteen minutes after running the voltammograms in each new sample. This high value of stabilisation time may be due to the complexation of catechol with other ions present in the solution such as copper and nickel. However, it was interesting to note that, after this stabilisation time, the response of the MFM at standard additions of iron was rapid and reproducible.

Collection potential

The voltammetric determination of iron in osteoblast-like cell culture medium using catechol suffers from interference from copper (in concentration ranging from $10^6$ to $10^7$ mol L$^{-1}$) which is present in the foetal bovine serum and in the sterile water used to prepare the cell culture medium and, forms a stronger complex with catechol than with iron producing an overlapping peak. van den Berg et al.\textsuperscript{16} suggested that EDTA must be added to the solution to mask the copper peak or to select a collection potential more negative than -0.25 V to promote dissociation of adsorbed complex ions of copper. Both alternatives were tried, however, without success. A high decrease in iron sensitivity was observed when EDTA was added in concentrations enough to mask copper. The
effect of the collection potential on the peak height (Fig. 1) was evaluated by ranging the potential from 0.00 V to -2.00 V. It was observed that overlapping of iron and copper peaks occurs when collection potentials of 0.00 V to -0.30 V and of -1.30 V to -1.60 V were used. When accumulation was carried out between -0.40 V and -1.00 V the sensitivity for iron diminished significantly as only a small peak or no peak was observed. This marked decrease may be caused by the variation of the electrode charge with respect to the solution. However, improved sensitivity for copper was observed by using a negative collection potential between -1.10 V and -1.30 V since for these values only the copper peak was present in the CSV scan. It is clear from Fig.1 that iron can only be measured selectively in culture medium digests if accumulation is carried out at collection potentials more negative than -1.60 V. Considering that well-defined peak separation between copper and iron (the copper and the iron peak appeared at -0.22 V and -0.42 V, respectively) was obtained for a collection potential of -1.80 V, this value was chosen as being the most suitable. The accumulation process of iron changed from a simple adsorption at deposition potentials more positive than ca. -0.42 V (which corresponds to the peak potential for the iron-catechol complex) to amalgamation followed by adsorption of iron when more negative potentials were used. The same pattern of behaviour was already observed by Capodaglio et al.\textsuperscript{32} in the determination of antimony in sea water with catechol. The amalgamated iron is re-oxidised to Fe\textsuperscript{3+} (complexed by catechol) and subsequently re-adsorbed when the potential is set to -0.10 V (or -0.05 V) to begin the CSV scan. It is quite clear that this procedure enables that only small amounts of iron are re-oxidised and complexed with catechol since re-adsorption occurred only during the period between switching to the scan start potential, -0.10 V, and scanning until the reduction potential of iron (ca. -0.42 V) had been
reached. Bearing this in mind, other experiments were carried out in which the re-oxidation step was performed separately before the CSV scan and both re-oxidation potential (0.00 V to -0.40 V) and time were varied. In some cases, peak overlap was again observed. Thus, it was decided to limit the re-adsorption step to the time of scanning.

![Graph of current (mA) vs. potential (V) showing three curves labeled a, b, and c.](image)

Figure 1- Effect of the collection potential on the iron peak: (a) -0.10 V; (b) -1.50 V and (c) -1.80 V.


**pH and buffer**

The main factor influencing peak shape, peak height, peak position and hence the peak-to-peak separation for iron and copper was the solution pH. The effect of pH was investigated in a sample containing $2.69 \times 10^{-6}$ mol L$^{-1}$ of iron, $1.89 \times 10^{-7}$ mol L$^{-1}$ of copper and $5.00 \times 10^{-4}$ mol L$^{-1}$ catechol whose pH was adjusted over the range of ca. 2.4 to 10. Fig. 2 shows the influence of pH in iron peak resolution. The iron peak became well separated from the copper peak at a pH value of ca. 7.3 since as the pH of the solution increases the iron peak moves away from the copper peak and overlapping is avoided. The CSV peak height of iron increased with the pH between 7.3 and ca. 8.5 as already has been reported by Torrance *et al.*$^{33}$ and for higher values of pH, the reduction current decreases possibly caused by competitive hydrolysis of the Fe$^{3+}$ ions$^{16}$. It was found also that the peak potential was strongly affected by the pH: a shift in a more negative direction of ca. 60 mV per unit pH was observed as a consequence of the greater stability of the iron-catechol complex ions at higher pH values$^{7}$. A working pH value of 8.0 was selected since good peak separation and sensitivity was attained. All experiments were carried out in $1.00 \times 10^{-2}$ mol L$^{-1}$ PIPES buffer. This buffer was chosen since it is an effective pH buffer to fix pH at 8.0, and does not interfere with the analysis of iron in culture medium digests. Other buffers were not tested since PIPES was considered the most suitable, from a practical point of view, since it will be possibly used to determine the total chromium content in osteoblast-like cell culture medium accordingly with the procedure described by Wang *et al.*$^{34}$ taking into consideration that chromium is a species associated with the biocompatibility evaluation of stainless steels biomaterials.
Figure 2- Effect of pH solution on the cathodic peak of iron: (a) pH=2.4; (b) pH=6.9 and (c) pH=8.5.

Complexing agent

The effect of varying the catechol concentration from 1.00x10^{-6} \text{ mol L}^{-1} to 1.00x10^{-2} \text{ mol L}^{-1} and the deposition time on the iron CSV peak height were investigated in several samples. Similar results as those reported in the literature^{16,33} were obtained. The catechol concentration and the maximum deposition time used in all experiments were 5.00x10^{-4} \text{ mol L}^{-1} and 30 s, respectively.
Linear range, detection limit, accuracy and precision

A sample containing $2.00 \times 10^6$ mol L$^{-1}$ of iron, the standard additions method and a deposition time of 30 s were used to establish the linear range in osteoblast-like cell culture medium digests using a MFM. The peak height increased linearly ($r=0.999$) with the iron standard additions until a final concentration of $1.54 \times 10^{-5}$ mol L$^{-1}$ was reached. Obviously, this linear range can be extended by using a shorter deposition time. It should be mentioned that the sensitivity (peak current/concentration) obtained with additions of Fe$^{2+}$ is practically the same as the one obtained with increments of Fe$^{3+}$. This feature enables us to quantify total iron$^{16}$.

Four determinations of the blank (using an accumulation time of 30 s at -1.80 V and six standard additions of iron) produced a relative standard deviation of 1.70%. The detection limit in these conditions, calculated from three times the standard deviation of the blank, as recommended by IUPAC$^{35}$, produced a value of $1.93 \times 10^{-8}$ mol L$^{-1}$.

The accuracy of iron determination was tested by comparison of results obtained by CSV with values attained by AAS. In both techniques, the iron content was quantified by using three standard additions with three replicates at each concentration (an example of a CSV quantification is presented in Fig. 3). The precision was evaluated by analysing the same sample three times. The values obtained by CSV -$(7.73 \pm 0.13) \times 10^{-6}$ mol L$^{-1}$; $(12.4 \pm 0.40) \times 10^{-6}$ mol L$^{-1}$; $(15.4 \pm 0.030) \times 10^{-6}$ mol L$^{-1}$- and AAS -$(7.61 \pm 0.080) \times 10^{-6}$ mol L$^{-1}$; $(12.9 \pm 0.73) \times 10^{-6}$ mol L$^{-1}$; $(15.3 \pm 0.12) \times 10^{-6}$ mol L$^{-1}$-.
differed only by 0.68-3.8% and the overall precision was good namely, 0.19-3.2% for CSV and 0.80-5.6% for AAS.

**Figure 3**- Voltammograms obtained by three standard additions of 7.16x10^{-7} mol L^{-1} of iron in osteoblast-like cell culture medium digest containing 4.66x10^{-7} mol L^{-1} of iron. The conditions were: collection time=30 s at -1.80 V.
**Biological effects**

In Fig. 4 it is illustrated the effect caused by the stainless steel concentration of $1.00 \times 10^{1}\%$ in osteoblast-like cell cultures *versus* the control cell culture. The experimental evidence indicates that the mineralisation process is slower in the presence of the highest concentration of stainless steel corrosion products when compared to the control and to all the other iron salt concentrations$^{36}$. This conclusion is in agreement with the results obtained by histochemistry (assessment of calcium and phosphate deposits). On the other hand, the biochemical assays (cell viability and alkaline phosphatase activity) showed that the osteoblast-like cells during the 28 days of assays were not always significantly (using the student’s $t$ distribution no significant difference was observed at 95% confidence level) affected by the exposure to the investigated concentrations of AISI 316L stainless steel corrosion products and iron salt solutions.
Figure 4- 21 days old osteoblast-like cells obtained from rabbit bone marrow (magnification X100) and grown in the presence of: (A) cell culture medium and (B) cell culture medium containing the highest concentration of stainless steel corrosion products.
CONCLUSIONS

In this work, an accurate electrochemical procedure was developed to determine iron in osteoblast-like cell culture medium using a MFM and catechol as the complexing agent. It is of interest to point out that this has been the first attempt, at least known to the authors, in which MFM coupled with adsorptive CSV has provided quantitative information about metal ions levels (e.g. iron) released from stainless steels in cell culture medium, envisaged to evaluate in vitro the mineralisation process which occurs at metallic biomaterials surface upon its implantation in living systems. The major differences between this procedure and the one presented by van den Berg et al.\textsuperscript{16} are the accumulation scheme and the pH value. These parameters had to be optimised due to the high content of copper present in the cell culture medium. The limit of detection obtained is adequate for iron quantification in the culture medium and in almost all biological materials (blood, body fluids, kidney, liver, spleen, lung, muscle, etc.) since iron is the most abundant heavy metal in animals\textsuperscript{37}. So, this procedure can be also used to examine tissues from humans and laboratory animals exposed to implant materials (in vivo analyses) providing valuable information about the dissemination of iron, as a corrosion product, throughout the body. The higher value of the limit of detection when compared with the one reported previously (6.00x10\textsuperscript{-10} mol L\textsuperscript{-1})\textsuperscript{16}, may be due to the very short re-oxidation time used (justified by the strong interference of copper).

Altogether, the biological results showed that stainless steel corrosion products, in the range of concentrations studied, did not interfere significantly in the process of in
vitro bone formation, i.e. in the mineralisation process. Interesting was the fact that corresponding concentrations of metal ions used separately were able to increase the cell viability. These distinct biological effects caused by corrosion products and corresponding metal ions constitute a matter of further investigation which is currently being performed at our laboratory.

REFERENCES


2.2- Nickel Determination in Osteoblast-Like Cell Culture Medium by Adsorptive Cathodic Stripping Voltammetry With a Mercury Microelectrode

ABSTRACT

The purpose of this study was to investigate the influence of nickel, which is an alloying element in commonly used metallic biomaterials, on the mineralisation process. An electrochemical method was developed to quantify this metal ion in osteoblast-like cell culture medium (OST) by performing adsorptive cathodic stripping voltammetry (CSV) with dimethylglyoxime (DMG) using a mercury film microelectrode (MFM). The optimised analytical conditions and the square-wave voltammetric parameters for the CSV analysis are: DMG concentration of $5.00 \times 10^{-4}$ mol L$^{-1}$, ammonium chloride buffer 0.100 mol L$^{-1}$ (pH=9.2), frequency 50 Hz, amplitude 20 mV, step 2 mV, adsorption time 10 s, deposition potential -0.70 V (vs. Ag/AgCl) and reduction potential -1.20 V. The limit of detection was $7.70 \times 10^{-9}$ mol L$^{-1}$ for an adsorption time of 10 s. The results achieved by CSV using the MFM were compared to those obtained by atomic absorption spectrometry (AAS) to ensure the reliability of the electrochemical method. The mineralisation process was evaluated by biochemical and histochemical assays.
INTRODUCTION

AISI 316L stainless steel (61-68% Fe, 17-20% Cr, 13-16% Ni and 2.3-3.5% Mo) is a commonly used biomaterial in orthopaedic surgery. The biocompatibility of metallic implants is controlled by the chemical, or more precisely by the electrochemical interaction that results in the release of metal ions into the tissue, and the toxicology of these released debris\(^1\). Although no specific function for nickel (one of the stainless steel corrosion products) has been established, evidence exists that is an essential metal to animals and humans\(^2\) which occurs at trace concentrations. At higher concentrations, it has been associated with the appearance of contact dermatitis, fibrosis, and of most concern, it was found to be carcinogenic\(^3,4\) and, to promote malignant fibrous histiocytomas at the site of metal implants containing nickel\(^5\).

Several authors have claimed and proved that the use of mercury film electrodes is advantageous over the use of HMDE\(^6-11\). The application of these electrodes to determine nickel in biological samples after oxidative acid treatment\(^11\) or in simple aqueous solution\(^12\) in combination with the inherent properties of microelectrodes\(^13\) was shown to be a reliable and promising technique\(^14\).

The main goal of our research team is to investigate the biodegradation and mineralisation process which occurs at surface of metallic biomaterials used in medical devices (e. g. AISI 316 L stainless steel and titanium) either by performing in vitro or in vivo studies\(^15-17\). Herein osteoblast-like cells, which are the cells that promote bone
growth and are used as cell culture test models to evaluate the biocompatibility of biomaterials, were grown in the presence of a corrosion product released from AISI 316L stainless steel, i.e., nickel in order to study its effects on the mineralisation process. The cellular evolution was monitored by performing biochemical and histochemical tests and by careful analysis at an inverted optical microscope. To quantify nickel levels in OST, an adsorptive CSV procedure using a MFM and DMG as the complexing agent was optimised. The adsorptive stripping voltammetry approach constitutes an useful alternative to the traditional method of AAS for studying the clinical performance of biomaterials since its high sensitivity (allowing large dilutions) associated with its non-destructive character reduces the old problem of medical research: too many biological and chemical parameters to quantify for the small amount of biological fluid or tissue available.

MATERIALS AND METHODS

Reagents

Osteoblast-like cell culture reagents

The OST consisted of α-MEM (α-Minimal Essential Medium SIGMA M 0894) supplemented with 10% of antibiotic antimycotic solution 10 times concentrated (solution with 1000 units of penicillin per mL, 10 mg/mL of streptomycin and 25 μg/mL of amphotericin B), 10% of foetal bovine serum, $10^{-8}$ mol L$^{-1}$ of dexamethasone, $10^{-2}$ mol L$^{-1}$ of β-glycerophosphate and $2.52 \times 10^{-4}$ mol L$^{-1}$ of ascorbic acid. α-MEM is a
buffered solution of pH 7.2 ± 0.3 containing essentially inorganic salts (calcium chloride, magnesium sulfate, potassium chloride, sodium chloride, sodium phosphate monobasic and sodium bicarbonate), amino acids and vitamins.

A salt solution of NiNO₃ containing 1.72x10⁻³ mol L⁻¹ of nickel was prepared in Hank's Balanced Salt solution (HBSS) which simulates the composition of the physiological extracellular fluids.

All reagents used were of suprapure (mineral acid, Merck) or analytical reagent grade (Merck). Deionised and triply distilled water was used for preparing all solutions.

**Procedures**

**Sample preparation**

Osteoblast-like cells were obtained from rabbit tibia bone marrow and their culture was performed according to the procedure reported by Maniatopoulos *et al.*¹⁸. After pH adjustment to 7.4 and sterilisation in a pressurised autoclave at 135°C during 20 min., the nickel solution was added to the cell cultures, except to those used as control, in three concentrations namely 1.72x10⁻⁶ mol L⁻¹, 1.72x10⁻⁷ mol L⁻¹ or 1.72x10⁻⁸ mol L⁻¹. These concentrations were chosen since they correspond to the content of nickel present in a metallic slurry of stainless steel corrosion products which biocompatibility was previously studied¹⁹ and more evidence about the effects promoted by nickel was searched in this study. Cells were incubated in a humidified atmosphere with 95% air and 5% CO₂ at 37°C and were sacrificed at days 7, 14, 21 and 28 of culture to perform
the biochemical and histochemical assays. The culture media were changed twice a week, collected and stored at -20°C.

Quantification and electrochemical studies of OST required mineralised samples. Digestion of samples were carried out in a microwave oven (CEM Model MDS-2000). After the digestion period, the decomposition solution was evaporated to dryness and the residues were dissolved in a volume of deionised and triply distilled water equal to the initial volume of the sample before destruction of the organic matter. This procedure was performed either for electrochemical Ni measurements and AAS Ni analysis.

*Mercury microelectrode preparation*

The working electrode was a $25 \times 10^{-6}$ m diameter gold microdisk. The MFM was prepared by electrodeposition of a mercury film onto a gold microdisk electrode by the application of a constant potential of 0.00 V during a deposition time of 60 s from a deoxygenated solution containing 1.00 mol L$^{-1}$ KNO$_3$ plus 5.70x$10^{-3}$ mol L$^{-1}$ Hg(NO$_3$)$_2$ and 0.500% concentrated nitric acid$^{20}$. The growth of the mercury films was monitored by the chronoamperometry technique and a linear relation between the current and time$^{32}$ was observed indicating that the process is mass transfer controlled and that nucleation is progressive$^{21-23}$.

*Voltammetric studies*

The voltammetric studies were performed with an AUTOLAB potentiostat/galvanostat model PSTAT 10 coupled with an ECD Module (Eco Chemie),
a working MFM, an Ag/AgCl reference electrode and a cylindrical carbon counter electrode.

For the CSV quantification of nickel in the OST both DMG (0.100 mol L$^{-1}$) and ammonia/ammonium chloride buffer (2.00 mol L$^{-1}$) were added to the sample, which was previously diluted at least six times, to give a final concentration of 5.00x10^{-4} mol L$^{-1}$ and 0.100 mol L$^{-1}$ (pH 9.2), respectively. The pre-concentration was carried out at -0.70 V during 10 s and the potential applied to remove adsorbed nickel-DMG complexes from the mercury film was -1.20 V during 30 s. The square-wave parameters used for the nickel quantification were a frequency of 50 Hz, an amplitude of 20 mV and a staircase step of 2 mV.

Measurements of nickel by the technique of AAS were made with a Zeeman Atomic Absorption Spectrometer Model 4100 ZL from Perkin Elmer in conjunction with a graphite tube furnace.

**RESULTS AND DISCUSSION**

Suitable microelectrodes for determination of nickel were previously reported as being carbon-fiber microelectrodes modified with conductive polymeric films$^{24}$ and MFM$^{8,14,18,24-31}$. Malinski et al. had developed a chemically modified microelectrode with a porphyrin film to determine nickel in single biological cells$^{24}$. Apparently, this method seems perfect. However, it can not be applied in this biocompatibility study
since the linear range \((i.e. \ 5 \times 10^{-3} \ \text{mol} \ \text{L}^{-1} \ \text{to} \ 5 \times 10^{-6} \ \text{mol} \ \text{L}^{-1})\) is too high; exposure of the osteoblast-like cells at such nickel concentrations would promote death of cells and consequently the nickel biochemical effects with time could not be investigated. So, it was decided to use a gold microdisk electrode modified with a mercury film, \(i.e.,\) a MFM\(^{32}\). The combination of a MFM with square wave voltammetry which minimise oxygen interference\(^{33}\) enabled to work without deoxygenation of the samples, without forced convection during the deposition step and also without equilibration period before the cathodic scan was started.

The CSV technique for nickel determination using DMG as complexing agent has been widely described by different experimental approaches and techniques\(^{11-12,14,34-40}\). However, in order to test the effect of the biological matrix on the stripping process, a series of optimisation studies was made. Some of the most relevant parameters are presented herein.

**Reproducibility**

To investigate the reproducibility of the response of the MFM in OST digests, fifty voltammograms of the same solution were runned during \(\text{ca.} \ 2.5 \ \text{hours}\). The worst reproducibility was observed during the first five scans \((-3.18 \pm 0.16 \ \text{nA})\) and for the next forty five scans a value of \(2.75\% \ (-2.66 \pm 0.070 \ \text{nA})\) was obtained for the variation of peak height measurements. Therefore, in all experiments executed with the MFM, the first ten voltammograms made with each new film were ignored.
Sample treatment

CSV investigations (Fig. 1) of nickel in OST after the digestion procedure indicated that mineralisation under the conditions of a conventional pressure decomposition with nitric acid leads, even under optimised conditions, to the formation of residues of organic compounds. The oxidation potential is not sufficient to digest all organic compounds completely\(^{41}\) and it was shown that these residues are formed exclusively from amino acids (phenylalanine, histidine, tryptophan and methionine) and linoleic acid present in significant concentrations in biological samples\(^ {42}\). The interference signal (peak A in Fig.1) during the voltammetric elemental analysis that appeared around -0.60 V was attributed mainly to the nitrobenzoic acids formed from phenylalanine and only to a slight extent from tryptophan reaction products. None of the other substances present in the biological materials made any appreciable contribution to the interference signal, as they are either completely degraded or present in such low concentrations that the interference signals of their reaction products are not hindered\(^ {43}\). Other substances, such as methionine and linoleic acid, yield products which are voltammetrically inactive in the potential range studied\(^ {43}\). The voltammetric examinations of all the biological samples containing protein with the application of square-wave voltammetry technique led to an identical interference signal\(^ {43}\). To eliminate this interference peak, the decomposition solution had to be evaporated to fumes with perchloric acid\(^ {41}\). This additional step increased, however, the time of sample treatment and the risk of introduction of contaminants. Bearing this in mind, together with the fact that the interference (peak A) and nickel (peak B) peaks are well separated and that sample dilution was always performed, it was decided not to perform this step and consequently
to work with decreased sensitivity due to the presence of the organic residues. A linear relationship between the stripping signal of nickel, that appeared around -0.99 V, and the concentration of metal dimethylglyoxime complex in the sample and after standard additions was observed.

![Graph](image)

**Figure 1**- Cathodic stripping voltammogram of the digested solution of osteoblast-like cell culture medium showing: (A) the interfering signal of organic residues and (B) the nickel peak obtained with the MFM.

*Influence of complexing agent concentration*

The effect of DMG on the nickel peak current (Fig. 2) had been reported previously for the HMDE$^{44}$ and similar results were obtained with the MFM.
The addition of DMG in the range of 1.00x10^{-6} - 1.00x10^{-3} mol L^{-1} promoted the decrease of the peak height of the interfering signal in 13.8%. It appeared that the complexing agent displaced some nickel bound to organic compounds. Complete formation could be expected to occur at a DMG concentration of 5.00x10^{-4} mol L^{-1} since for higher ligand concentration the peak current of the interfering signal remained constant. Thus a DMG concentration of 5.00x10^{-4} mol L^{-1} was used in all the measurements.

![Graph showing the dependence of peak current of nickel and interfering signal on DMG concentration.]

**Figure 2-** Dependence of peak current of nickel (A) and of the interfering signal (B) on the concentration of DMG. Conditions: deposition time=10 s; deposition potential=-0.70 V (vs. Ag/AgCl); [NH_{4}NH_{4}Cl]=0.100 mol L^{-1} (pH=9.2); [nickel]=1.16x10^{-6} mol L^{-1}; frequency=50 Hz; amplitude=20 mV and step=2 mV.
Effect of the frequency

This parameter had a different effect in the interfering signal and in the nickel peak. An increase of the current height was observed up to ca. 100 Hz and ca. 200 Hz for the organic residues peak and nickel peak, respectively. Then, the nickel peak became ca. constant and the interference peak current started to decrease sharply indicating that at high scan rates the highly specific and very strong nature of the nickel-DMG complex was preferentially adsorbed\textsuperscript{45}.

A square-wave frequency of 50 Hz was adopted for the Ni measurements in OST digests, being a compromise between the peak current of the interfering signal and sloping background current which was observed for higher frequencies and, rendered the measurements difficult due to the significant contribution of the capacitive current\textsuperscript{46}.

Linearity range, detection limit, accuracy and precision

A sample containing 2.03x10\textsuperscript{-6} mol L\textsuperscript{-1} of nickel and the standard additions method were used to calibrate the CSV sensitivity in the samples of OST digests (Fig. 3). With a 5 s adsorption time the plot was found to be linear (r=0.999) up to the eighth standard addition of nickel which corresponded to a final concentration of 3.41x10\textsuperscript{-6} mol L\textsuperscript{-1} in the sample. When further standard additions were made, deviations from linearity became significant due to the saturation of the MFM.
Figure 3. The CSV peak current as a function of nickel concentration in osteoblast-like cell culture medium digest containing $2.03 \times 10^{-6}$ mol L$^{-1}$ of nickel. Conditions: deposition time=5 s; deposition potential=-0.70 V (vs. Ag/AgCl); [DMG]=$5.00 \times 10^{-4}$ mol L$^{-1}$; [NH$_3$/NH$_4$Cl]=$0.100$ mol L$^{-1}$ (pH=9.2); frequency=50 Hz; amplitude=20 mV and step=2 mV.

The detection limit was calculated from three times the standard deviation of the blank, as recommended by IUPAC and a value of $7.70 \times 10^{-9}$ mol L$^{-1}$ was obtained for a deposition time of 10 s. It should be noted that the presence or absence of oxygen in the sample did not influence the detection limit for nickel when MFM are used in combination with square-wave voltammetry.
The accuracy of the proposed procedure to determine nickel in OST digests was tested by comparison of the results obtained by CSV with the values obtained for the same samples but using AAS (Table 1). In both techniques, the nickel content was quantified by using five standard additions with three measurements at each concentration (an example of a CSV quantification is presented in Fig. 4) and the precision was tested by analysing three times the same sample. As it can be seen from Table 1, there is a good agreement between the results obtained by CSV and AAS which indicate that the presence of organic residues in the digest samples did not affect the quantification of nickel using a MFM, DMG as the complexing agent and the standard addition method which eliminated the matrix effect. Similar results were obtained by Bond *et al.* in natural waters of Southern Australia using the HMDE. This fact was attributed to the very strong and specific nature of the nickel complexation by DMG \((\log \beta = 17.24^4)\) which competes with organic ligands to bind preferentially to nickel. The values obtained by CSV and AAS differed only by 0.070-0.99% and the overall precision was quite good namely, 0.20-2.2% for CSV and 0.12-1.3% for AAS.
Table 1- Comparison of measured values of nickel in the cell culture medium containing the highest concentration of the nickel salt obtained by CSV and AAS.

<table>
<thead>
<tr>
<th>Days of culture</th>
<th>Nickel concentration/10^7 mol L(^{-1}) CSV</th>
<th>AAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>27.8 ± 0.62</td>
<td>27.7 ± 0.030</td>
</tr>
<tr>
<td>14</td>
<td>12.1 ± 0.18</td>
<td>12.1 ± 0.16</td>
</tr>
<tr>
<td>25</td>
<td>38.7 ± 0.080</td>
<td>38.7 ± 0.20</td>
</tr>
<tr>
<td>28</td>
<td>48.0 ± 0.90</td>
<td>47.5 ± 0.28</td>
</tr>
</tbody>
</table>

Figure 4- Voltammograms obtained by five standard additions of 1.70x10\(^{-7}\) mol L\(^{-1}\) nickel in osteoblast-like cell culture medium digest containing 2.41x10\(^{-7}\) mol L\(^{-1}\). Conditions: deposition time=10 s; deposition potential=-0.70 V; [DMG]=5.00x10\(^{-4}\) mol L\(^{-1}\); [NH\(_3\)/NH\(_4\)Cl]=0.100 mol L\(^{-1}\) (pH=9.2); frequency=50 Hz; amplitude=20 mV and step=2 mV.
Optimisation of the adsorption potential, adsorption time (no significant influence on the interference peak was observed), buffer concentration, pH, amplitude and staircase step gave similar results as those obtained by previous studies\textsuperscript{34,13,48,33}. A buffer concentration of 0.100 mol L\textsuperscript{-1} (pH=9.2); an adsorption potential of -0.70 V, an adsorption time between 5 s and 30 s, an amplitude of 20 mV and a staircase step of 2 mV were selected as the optimum values.

\textit{Biological effects}

From Table 1, it can be observed that during the first 14 days of cells cultivation there is a decrease of nickel contents in the OST, indicating that nickel is somewhat involved in the cell growth since it is during this period that the faster proliferation rate of osteoblast-like cells occurs (the growth rate of this type of cells is characterised by an exponential phase followed by a stationary phase). Thus, from day 14 to 28, the osteoblast-like cells presented a steady growth and nickel levels are kept higher in the OST.

In Fig.5 it is illustrated the normal polygonal appearance of the osteoblast-like cells (Fig. 5A) and the cell morphology in the presence of the nickel salt (Fig. 5B) at day 7th. It can be observed that, in the later case, some osteoblast-like cells had an abnormal shape as well as aspect and, deposits of the metal ion can be observed across the cell culture. However, the biochemical results indicated that during the 28 days of exposure to the Ni, the cells increased their cell viability (they proliferated more rapidly than in normal conditions) but modified their phenotypic expression since a decrease in an
osteoblast marker, the alkaline phosphatase activity (ALP), was observed. Since ALP plays a crucial role in the initiation of the mineralisation\textsuperscript{49}, this decrease indicated that a defective bone formation, or at least a lag in the mineralisation process, will occurred when nickel alone is released from metallic implants.
Figure 5- 7 days old osteoblast-like cell culture obtained from rabbit bone marrow (magnification X200) showing the effect of nickel in the mineralisation process: (A) control culture and (B) culture grown in the presence of $1.72 \times 10^{-8}$ mol L$^{-1}$ of nickel.
CONCLUSIONS

In this work, the CSV characteristics of nickel in OST were investigated using a MFM. High reproducibility of stripping voltammograms (ca. 2.8%) was obtained by using a MFM. Due to the enhanced mass transfer attained when using microelectrodes, preconcentration of nickel was accomplished in quiescent solutions\textsuperscript{50} and no equilibration period was used before initiating the cathodic scan. The application of the square-wave voltammetry technique in conjunction with a MFM overcomes the need for deoxygenation of samples for nickel determination. Interference from residual organic matter was eliminated by the optimisation of operating parameters and by using the standard addition method in diluted samples. It was also shown that stripping voltammetry is an attractive method for routine metal quantification in biological samples due to its high sensitivity and accuracy, non-destructive character, short analysis time and low cost. An important fact that may be of interest to point out, is that the presence of large quantities of salt components for voltammetry is usually rather an advantage than a problem in contrast with AAS where signal suppression by matrix interferences may occur\textsuperscript{51}.

It was interesting to note that the exposure of cells to nickel ions caused an increase in cell viability throughout the 28 days of study in contrast with what happens in the presence of a prepared physiological slurry containing the corrosion products of AISI 316L stainless steel since, in this case, the cell viability was not significantly affected\textsuperscript{49}.  

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2.3- Chromium Determination in Osteoblast-Like Cell Culture Medium by Catalytic Cathodic Stripping Voltammetry With a Mercury Microelectrode

ABSTRACT

A catalytic cathodic stripping voltammetric procedure for the determination of total chromium in osteoblast-like cell culture medium using a mercury film microelectrode (MFM) was optimised. The method is based on the pre-concentration of the Cr(III)-diethylenetriaminepentaacetic acid (DTPA) complex by adsorption at the potential of -1.00 V (vs. Ag/AgCl) in the presence of 10.0x10⁻³ mol/L DTPA, 0.700 mol/L sodium nitrate, 4.00x10⁻² mol/L sodium acetate and 1.00x10⁻³ mol/L potassium permanganate at pH 5.9-6.0. The limit of detection obtained for a 40 s collection time was 2.80x10⁻¹⁰ mol/L of chromium. The results achieved by stripping voltammetry using the MFM were compared to those obtained by atomic absorption spectrometry (AAS) to ensure the reliability of the electrochemical method. This procedure proved to be an alternative to AAS and valuable in biocompatibility studies performed in vitro using osteoblast-like cells.
INTRODUCTION

Clinical reports, controlled in vivo animal experiments and in vitro cell culture methods have been definitive in demonstrating the release, transport and absorption of corrosion products from implanted AISI 316L stainless steel (a metallic alloy widely used in orthopaedic surgery)\(^1-9\). Increasing attention is rightly being focused on the biological consequences caused by such burden since metabolic, bacteriologic, immunologic and carcinogenic effects have been proposed\(^10-12\). These considerations suggest the importance of the investigation of changes in concentration of metals associated with metallic implantation.

Chromium has been determined in water using anodic stripping voltammetry\(^13\), polarography\(^14\) or cathodic stripping voltammetry (CSV)\(^15-18\). This last approach, based on the adsorptive collection of the complex of chromium (III) with DTPA\(^15-16\), with triethylenetetraminehexaacetic acid (TTHA)\(^17\) or with cupferron\(^18\) on a hanging mercury drop electrode, constitutes the most sensitive procedure. While trace-metal determination in natural waters is rapidly becoming the domain of the voltammetric methods, due to their excellent sensitivity which is not bettered by any non-electrochemical method for the metals of particular toxicological interest\(^19\), the analytical techniques commonly used for metal determination in biological materials are still AAS and neutron activation analysis\(^20\). However, previously described applications of the adsorptive stripping voltammetry, which have proved to be very successful, for iron\(^21\) and nickel\(^22\) determination in in vitro AISI 316L stainless steel biocompatibility
cell culture tests in combination with the reported sensitivity for chromium in natural waters\textsuperscript{15-18}, encouraged us to optimise the method developed by J. Golimowski \textit{et al.}\textsuperscript{15} for further chromium determination in cell culture medium using a MFM. Chromium quantification associated with iron\textsuperscript{21}, nickel\textsuperscript{22} and calcium\textsuperscript{23} measurements enable to study the variation of metal content in bone-cell cultures exposed to AISI 316L stainless steel corrosion products, and in conjunction with histochemical, morphological and biochemical assays, to characterise dose- and time-effects of degradation products in \textit{in vitro} bone cell (osteoblast-like cell) behaviour and in the mineralisation process (biologically induced deposition of calcium phosphates which occurs during bone formation)\textsuperscript{24-26}. The achieved results are discussed herein. The accuracy of the electrochemical procedure for chromium quantification was ensured by comparison of the results obtained by CSV with those obtained for the same samples but using AAS.

\textbf{MATERIALS AND METHODS}

\textit{Sample preparation}

Rabbit bone marrow stromal cells (obtained accordingly with the procedure described previously\textsuperscript{24}) were seeded into multi-well culture plates and grown in conditions known to favour the formation of osteoblast-rich cell cultures namely, in the presence of 2.52x10\textsuperscript{-4} mol/L ascorbic acid, 10\textsuperscript{-2} mol/L β-glycerophosphate and 10\textsuperscript{-8} mol/L dexamethasone\textsuperscript{24,27}. The cell culture medium\textsuperscript{24} consisted basically in a buffered solution of pH 7.2 ± 0.3 containing inorganic salts (calcium chloride,
magnesium sulphate, potassium chloride, sodium chloride, sodium phosphate monobasic and sodium bicarbonate), 21 amino acids, 11 vitamins, foetal bovine serum, antibiotic antimycotic solution, dexamethasone, β-glycerophosphate and ascorbic acid\textsuperscript{21-22}. An electrochemically prepared AISI 316L stainless steel stock slurry (8.95x10\textsuperscript{-3} mol/L Fe + 2.35x10\textsuperscript{-3} mol/L Cr + 1.72x10\textsuperscript{-3} mol/L Ni) was added, after pH adjustment to 7.4 and sterilisation in an autoclave, to the cell cultures, except to those used as control, in several concentrations namely, 1.00x10\textsuperscript{-1}\% , 1.00x10\textsuperscript{-2}\% or 1.00x10\textsuperscript{-3}\% (% relative to the stock slurry). Similarly, chromium salt (CrCl\textsubscript{3}) was added at equivalent concentrations, \textit{i.e.}, 2.35x10\textsuperscript{-6} mol/L, 2.35x10\textsuperscript{-7} mol/L or 2.35x10\textsuperscript{-8} mol/L. Cultures were incubated in a humidified atmosphere with 95\% air and 5\% CO\textsubscript{2} at 37\textdegree\ C and were sacrificed at days 7th, 14th, 21st and 28th to perform the biochemical and histochemical assays. Samples of culture medium were collected and stored at -20\textdegree\ C for further analysis\textsuperscript{24}.

Electrochemical studies and quantification of chromium in osteoblast-like cell culture medium required mineralised samples. Digestion of samples with concentrated nitric acid (suprapure 65\%) was carried out in a CEM Model MDS-2000 microwave oven. After the digestion period, the decomposition solutions containing stainless steel slurry were evaporated to fumes with perchloric acid\textsuperscript{21,28} and all the other were simply evaporated to dryness\textsuperscript{29}. The residues were dissolved in deionised and triply distilled water to the initial volume of the sample, \textit{i.e.} the volume before destruction of the organic matter. This procedure was done either for electrochemical measurements and for AAS analyses.
Voltammetric studies

All reagents used were of suprapure (mineral acid, Merck) or ACS reagents (at least >99%, Aldrich). Deionised and triply distilled water was used for preparing all solutions.

Standard solutions of Cr(VI) were prepared daily by dilution of a 1.93x10^{-2} mol L^{-1} Aldrich atomic absorption spectrometric standard solution.

The voltammetric studies were performed with an AUTOLAB potentiostat/galvanostat model PSTAT 10 coupled with an ECD Module (Eco Chemie), a working MFM, an Ag/AgCl reference electrode and a cylindrical vitreous carbon counter electrode. The MFM was prepared by electrodeposition of a mercury film onto a gold microdisk (radius=12.5x10^{-6} m; purchased from the Department of Chemistry of the University of Southampton) by the application of a constant potential of 0.00 V during a deposition time of 60 s, from a deoxygenated solution containing 1.00 mol L^{-1} KNO_{3} plus 5.70x10^{-3} mol L^{-1} Hg(NO_{3})_{2} and 0.500% concentrated nitric acid^{29}.

For the CSV analyses of chromium in osteoblast-like cell culture medium, DTPA (0.250 mol/L), sodium nitrate (5.00 mol/L), sodium acetate (4.00 mol/L) and potassium permanganate (1.00 mol/L) were added (reagents can be pre-mixed to minimise the pipetting during the reagent addition^{16}) to the sample, which was previously diluted with deionised and triply distilled water at least ten times, to give a final concentration of 10.0x10^{-3} mol L^{-1}, 0.700 mol L^{-1}, 4.00x10^{-2} mol L^{-1}, and 1.00x10^{-3} mol L^{-1},
respectively. The required pH value was adjusted to 5.9-6.0 by addition of aqueous solution of sodium hydroxide. Before the voltammetric analysis, the working MFM was inserted in the solution and then, the dissolved oxygen was removed by purging with nitrogen during 12 minutes. Similarly to previously reported studies\textsuperscript{21-22} and due to the inherent properties of microelectrodes\textsuperscript{30-31}, the pre-concentration was accomplished in quiescent solutions, at the optimal potential of -1.00 V during a deposition time between 5 and 40 s (depending on the chromium concentration in the sample), and no equilibration period was used before initiating the cathodic scan. The potential applied to remove adsorbed Cr(III)-DTPA complexes from the mercury film was -1.40 V during 5 s. The square-wave parameters selected for the chromium quantification were a frequency of 10 Hz, an amplitude of 20 mV and a staircase step of 3 mV.

Determination of chromium in the biological samples by the technique of AAS was performed with a Zeeman Atomic Absorption Spectrometer Model 4100 ZL from Perkin Elmer and using Mg(NO$_3$)$_2$ as the matrix modifier.

**RESULTS AND DISCUSSION**

Electroanalytical techniques, particularly adsorptive stripping voltammetric procedures, have been successfully developed for measuring chromium at trace levels in water samples using a chelating agent such as DTPA\textsuperscript{15-16}, TTHA\textsuperscript{17} or cupferron\textsuperscript{18}. The procedure described by Wang *et al.*\textsuperscript{18} is particularly attractive since it requires an easier sample preparation for measurement of total chromium content when compared to the
other methods, and also, in our specific case, it uses the same buffer (PIPES) as the one
need for iron determination in osteoblast-like cell culture medium\textsuperscript{21}. Regrettably, this
approach had to be discarded due to the high iron/chromium ratio existing in cell culture
medium exposed to corrosion products\textsuperscript{25-26} since cupferron is highly selective for iron.
No significant differences between results obtained with DTPA and TTTHA were
mentioned in previously reported comparative studies\textsuperscript{16}. In this study, the adsorptive
pre-concentration of Cr(III)-DTPA complexes at the MFM in osteoblast-like cell culture
medium was investigated in a sample containing $4.81 \times 10^{-8}$ mol/L of Cr(VI), in order to
test the effect of the biological matrix on the operational parameters, and applied to the
chromium determination.

**Sample treatment**

As reported previously\textsuperscript{22,32-33}, examinations of biological samples (containing
protein) decomposed with nitric acid led, even under optimised conditions\textsuperscript{33}, to a
voltammetric interference signal (Fig. 1) which may be attributed to the nitrobenzoic
acids formed during the digestion process mainly from two amino acids: phenylalanine
and tryptophan\textsuperscript{34}. To eliminate this interference peak that appeared around -0.35 V (at
pH 5.9-6.0), the digested solution had to be evaporated to fumes with perchloric acid\textsuperscript{33}. This additional step increases the time of sample treatment and the risk of introduction
of contaminants. However, for iron (major component of stainless steels which
evaluation is also useful in biocompatibility tests) quantification in osteoblast-like cell
culture medium, it is indispensable or peak overlap will occur (iron peak appeared at
ca. -0.42 V)\textsuperscript{21}. Bearing this in mind, together with the facts that i) the interference signal
(ca. -0.35 V, Fig. 1 peak A) and chromium peak (ca. -1.20 V, Fig. 1 peak B) are well separated, and ii) a ten fold sample dilution, at least, was always performed, it was decided to perform this step only in samples treated with stainless steel corrosion products (not with chromium salt) to allow for further iron quantification\textsuperscript{25-26}.

![Figure 1- Cathodic stripping voltammogram of the digested solution of osteoblast-like cell culture medium showing: (A) the interfering signal of organic residues and (B) the chromium peak obtained with the MFM.](image)

Since formation of Cr(III)-DTPA complexes from free Cr(III) ions (which are formed during the deposition step at -1.00 V since Cr(VI) is reduced to Cr(III) at potentials < -0.05 V) and DTPA proceeds instantaneously under voltammetric conditions\textsuperscript{35-36}, and also that total chromium content aimed to be determined, oxidation
of chromium species, existing in solution in undefined valence states, to Cr(VI) was performed before the analysis by adding potassium permanganate. This oxidant was added in excess (final concentration in solution 1.00x10^{-3} \text{ mol/L}) promoting that the pink colour remained for more than 5 min., which indicated complete oxidation of chromium compounds to Cr(VI)^{37-38}. Furthermore, conversion efficiency (and accuracy) was verified by comparison of results obtained by CSV and AAS for total chromium content (discussed below).

**Nitrate concentration**

The CSV scan of Cr(VI) in osteoblast-like cell culture medium digest produced a well defined peak at ca. -1.2 V due to the reduction of Cr(III) to Cr(II)^{15-16,39-40} which, in the presence of nitrate ions, is enhanced by a catalytic effect^{15-16,39-42}. Tanako et al.\textsuperscript{41} were the first research group to report that these increased currents were attributable to the chemical re-oxidation of Cr(II) to Cr(III) by nitrate, which is subsequently re-reduced at the electrode surface^{15-16,39-40}.

As the nitrate concentration increases the rate of Cr(II) → Cr(III) oxidation by nitrate is enhanced; a linear dependence (Fig. 2) was obtained until ca. 0.70 \text{ mol/L}, and thereafter, till it reaches a maximum at approximately 1.1 \text{ mol/L} the variation was non-linear. At higher oxidant concentrations, the peak current slightly diminished possibly due to the decrease of the adsorbed Cr(III)-DTPA at such high concentrations of electrolyte\textsuperscript{43}. A similar profile was obtained by M. Boussemart et al.\textsuperscript{16} in sea water studies.
It is interesting to point out that the current of the interference peak increased ca. 31% when the nitrate concentration was varied between 0-0.100 mol/L, and then remained approximately constant (variations of ca. ± 4% were observed) suggesting that the residues of organic complexes may have bind some chromium.

A concentration of 0.700 mol/L of nitrate was selected as a compromise between high sensitivity and low blanks.

Figure 2: Effect of varying the nitrate concentration on the CSV peak height of the interference (□) and chromium peaks (■) in cell culture medium digest containing 4.81x10^{-8} mol/L of chromium. The conditions were: pre-concentration during 30 s at -1.00 V.
**DTPA concentration**

Results presented in Fig. 3 show that the chromium peak current rose ca. 1.5 time when the complexing agent concentration was varied from $1.00 \times 10^{-3}$ mol/L to $10.0 \times 10^{-3}$ mol/L, remained approximately constant between $10.0 \times 10^{-3}$ mol/L and $15.0 \times 10^{-3}$ mol/L, and then started to decrease. The decay of the chromium peak height at high concentrations of DTPA may be caused by the formation of higher order complexes as Cr(III)-DTPA$_2^{15-16}$.

The pattern of variation obtained for the interference signal was quite different; only slight variations were observed till the addition of $7.50 \times 10^{-3}$ mol/L DTPA which promoted a significant current decrease of ca. 14% and then the interference peak remained approximately constant. It appeared that for DTPA concentrations equal or higher than $7.50 \times 10^{-3}$ mol/L, some chromium bound to the organic residues is displaced.

The optimal DTPA concentration selected (approximately the maximum and minimum current for chromium and interference peak, respectively) was $10.0 \times 10^{-3}$ mol/L which is much higher than the reported for distilled water ($5.00 \times 10^{-3}$ mol/L$^{15}$) and sea water ($2.50 \times 10^{-3}$ mol/L$^{16}$) probably due to the presence of residues of organic matter and to the competition for DTPA by cations such as iron, nickel, copper, calcium and magnesium existing in the cell culture medium at high concentrations (ca. $1.8 \times 10^{-5}$ mol/L Fe, ca. $4.9 \times 10^{-7}$ mol/L Ni, ca. $1.6 \times 10^{-7}$ mol/L Cu, ca. $2.7 \times 10^{-3}$ mol/L Ca and ca. $8.2 \times 10^{-4}$ mol/L Mg; without considering the addition of
corrosion products). Accordingly with previous reports\textsuperscript{16,44}, this may help to explain the lower sensitivity found in osteoblast-like cell culture medium, as compared to the reported for fresh\textsuperscript{15} and sea water\textsuperscript{16}. Nevertheless, a linear relation between the stripping signal was obtained when standard additions were made suggesting that matrix effect may be reduced by using the standard additions method.

![Graph](image)

**Figure 3.** Effect of varying the DTPA concentration on the CSV peak height of the interference (□) and chromium peaks (■) in cell culture medium digest containing $4.81 \times 10^8$ mol/L of chromium. The conditions were: pre-concentration during 20 s at -1.00 V.
**pH and acetate concentration**

The marked pH effect on the peak height of the Cr(III)-chelate reduction was evaluated over the pH range 5.0-7.1 (see Fig. 4) and similar results as those reported by other authors\(^{15,40,42}\) were obtained. The chromium peak current was maximal at pH ca. 6.0 as a consequence of the greater stability of the Cr(III)-DTPA complexes at this pH\(^{15}\), and was drastically decreased by a pH change of ±1.

The interference signal was affected differently since a trend for current increase was observed when the pH was varied between 5.0 and 7.1. This event was somewhat expected in view of the voltammetric behaviour of nitrobenzoic acid compounds with pH\(^{34}\).

Experiments were carried out at pH 5.9-6.0 in 4.00x10\(^{-2}\) mol/L sodium acetate. This concentration was chosen as being the more appropriate considering the baseline, chromium peak resolution and current height (the baseline became sharper and chromium peak height increased as the acetate concentration was varied from 0 to 0.100 mol/L at a constant pH of ca. 6.0).
**Figure 4** - Effect of varying pH on the CSV peak height of the interference (□) and chromium peaks (■) in cell culture medium digest containing 4.81x10⁻⁸ mol/L of chromium. The conditions were: pre-concentration during 20 s at -1.00 V.

*Adsorption potential and time*

Accordingly with previous reports¹⁵⁻¹⁶,⁴⁰ a marked dependence of the Cr(III)-DTPA reduction peak on the adsorption potential was observed. The chromium peak current doubled when the potential was increased negatively from -0.80 V to -1.00 V reaching a maximum at this value (from about -0.80 V onwards the accumulation of Cr(III)-DTPA complexes is enhanced by the nitrate ions).
The interference current height increased up to ca. -0.30 V and then, decreased until the onset of the reduction peak at ca. -0.35 V was reached, remaining constant thereafter.

The effect of accumulation time was investigated and it was observed that non-linearity for the chromium peak occurred for values longer than 40 s probably caused by the saturation of the MFM surface. No influence of the deposition time on the interference peak was verified.

For chromium analyses in osteoblast-like cell culture medium, the deposition potential and maximum collection time used were -1.00 V and 40 s, respectively.

**Instrumental parameters**

Optimisation of the square-wave parameters indicated that a frequency of 10 Hz, an amplitude of 20 mV and a staircase step of 3 mV were the more suitable for chromium quantification in cell culture medium digest taking in consideration the chromium peak definition and resolution, as well as the sensitivity. High scan-rates (>80 mV/s) promoted a decrease in the chromium sensitivity probably due to the poor electrochemical reversibility of the reduction step\(^{16}\). Also, it is worthwhile to note that square-wave voltammetry is advantageous when compared to the differential pulse mode, since the hydrogen ion reduction is irreversible and, thus, it is shifted to more negative potentials\(^{43}\).
Comparative optimisation studies carried out with the same sample but evaporated to fumes with perchloric acid gave generally similar results for the chromium peak variation, suggesting that the reduced sensitivity, as compared to water, is mainly due to complexation of DTPA by iron, nickel, copper, calcium and magnesium. Consequently, the same parameters and concentration of reagents were used for chromium determination in samples obtained from osteoblast-like cell cultures exposed to AISI 316 L stainless steel corrosion products and chromium salt.

Reproducibility

To investigate the reproducibility of the chromium peak, forty CSV scans of the same solution were runned during ca. 1 hour. The mean ± standard deviation obtained was (2.32 ± 0.040) nA.

For the Cr(III) response (no potassium permanganate was added to the sample), a time dependence of the current was observed since a peak height decrease of ca. 34% occurred in 30 min., justifying the selection of an oxidative pre-treatment of the sample. This peak instability may be caused by the conversion of the Cr(III)-complex to an electrochemically inert complex with time\textsuperscript{16}. Nevertheless, previous speciation studies showed that it is possible to determine the Cr(III) content by measuring the peak height produced immediately upon the addition of DTPA or calculate it by the difference between total chromium and Cr(VI) concentrations\textsuperscript{16}.
Linear range, detection limit, accuracy and precision

A sample containing 1.77x10^{-8} mol L^{-1} of chromium, the standard additions method and a deposition time of 5 s were used to establish the linear range in osteoblast-like cell culture medium digest using a MFM. The peak height increased linearly (r=0.9998) with the chromium standard additions until a final concentration of ca. 1.7x10^{-7} mol L^{-1} was reached.

Four determinations of the blank (using an accumulation time of 40 s at -1.00 V and three standard additions of chromium) produced a relative standard deviation of 3.73%. The detection limit in these conditions, calculated from three times the standard deviation of the blank, as recommended by IUPAC\textsuperscript{45}, produced a value of 2.80x10^{-10} mol L^{-1}.

The accuracy of chromium determination was tested by comparison of results obtained by CSV with those attained by AAS for the same set of samples. In both techniques, the chromium content was quantified by using three standard additions with three replicates at each concentration (an example of a CSV quantification is presented in Fig. 5). The precision was evaluated by analysing the same sample three times. The values obtained by CSV-(1.69±0.020)x10^{-6} mol L^{-1}; (2.45±0.070)x10^{-6} mol L^{-1}; (1.03±0.030)x10^{-6} mol L^{-1}; (5.40±0.060)x10^{-7} mol L^{-1} and AAS-(1.69±0.040)x10^{-6} mol L^{-1}; (2.39±0.020)x10^{-6} mol L^{-1}; (1.02±0.010)x10^{-6} mol L^{-1}; (5.58±0.12)x10^{-7} mol L^{-1}- differed only by 0.10-3.2% (no significant differences were found when comparing statistically the values obtained by both techniques using the
two-sided $t$-test at 95\% and 99\% confidence level) which confirm that the proposed electrochemical method is accurate and an alternative to AAS. Results also indicated that the presence of some organic residues in the digested samples did not affect the quantification of chromium using a MFM if optimal values for the operational parameters and the standard additions method are used. The overall precision was good namely, 1.1-2.9\% for CSV and 0.080-2.3\% for AAS.

![Voltammograms](image)

**Figure 5**- Voltammograms obtained by three standard additions of $9.62 \times 10^{-9}$ mol L$^{-1}$ of chromium in osteoblast-like cell culture medium digest containing $1.43 \times 10^{-8}$ mol L$^{-1}$ of chromium. The conditions were: pre-concentration during 20 s at -1.00 V.
CONCLUSIONS

Suitability of the CSV method for the determination of chromium, in biocompatibility studies performed in vitro with osteogenic cells, using a MFM was attained by optimisation of the operational parameters. The major differences, as compared with the determination in fresh water\textsuperscript{15} and sea water\textsuperscript{16} using the HMDE, are the higher nitrate and DTPA concentrations needed to reach a good sensitivity, and the shorter deposition times used (consequence of the microelectrodes properties). Ranking of matrices accordingly with sensitivity suppression, based on previous and present studies, would give: osteoblast-like cell culture medium digests $>$ sea water\textsuperscript{16} $>>$ fresh water\textsuperscript{15}. The previously higher detection limit obtained in sea water (ca. 0.1x10\textsuperscript{-9} mol/L), as compared with fresh water (ca. 0.01x10\textsuperscript{-9} mol/L), was attributed to the competition for DTPA by magnesium and calcium\textsuperscript{16}. Herein, decreased sensitivity resulted mainly (the presence of some organic residues seemed not to interfere significantly since no marked betterment was obtained by treating the sample with perchloric acid) from the presence of these cations (although in a lower concentration than the existing in sea water) and of iron, nickel and copper which are strongly complexed by DTPA ($\log\beta$=27.5 for Fe\textsuperscript{3+}, $\log\beta$=20.0 for Ni\textsuperscript{2+} and $\log\beta$=20.5 for Cu\textsuperscript{2+}\textsuperscript{46}). However, the limit of detection achieved (ca. 0.3x10\textsuperscript{-9} mol/L) is adequate for in vitro and in vivo chromium analyses of biological fluids and materials (chromium concentration in serum, blood clot and urine\textsuperscript{47,48} as well as in kidney and liver\textsuperscript{49} change after total hip replacement) and also of cells (exposed in vitro to corrosion products or collected in loco from the vicinity of a metallic implant). Furthermore, the procedure
described herein associated with the previously developed for iron
to nickel and calcium quantification in cell culture medium proved to be valuable tools in this biocompatibility study performed with cells of rabbit origin, as well as in other two tests based on rat and human osteoblast-like cells. Briefly, and analysing globally the results of the three assays, it seems that the Cr(III) salt did not reproduce the biological behaviour of chromium compounds that originate from AISI 316L corrosion products since substantial reductions (ca. 42% to 65%) in the chromium level in cell culture media treated with corrosion products and exposed to cells were measured in contrast with the slight and not significant variations (ca. 10-17%) that occurred in cultures exposed to Cr(III) salt. Cr(VI) is able to penetrate the cell membrane, but not trivalent chromium which is excluded (except at concentrations 100 or 1000 times those for the hexavalent form) and binds preferentially to serum proteins. These observations suggest that chromium resulting from AISI 316L corrosion products exists in the presence of bone cells, partially or totally, in the hexavalent state which could help to explain the higher cytotoxicity of AISI 316L corrosion products as compared with Cr(III) salt (at the concentrations studied). Metal species react in biological systems and as different species are formed, significant changes in toxicity occur. The stripping voltammetry approach has the particular advantage of being, in principle, a species sensitive method, not just an element sensitive method as the traditional technique of AAS. In loco measurements, performed without any chemical pre-treatment, constitute the major and future challenge of the microelectrodes technique in the biomedical field.
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2.4- Potentiometric Determination of Total and Ionised Calcium in Osteoblast-Like Cell Culture Medium

ABSTRACT

The effect of the ionic strength and composition of solutions in the direct potentiometric determination of total and ionised calcium content using a calcium selective electrode was investigated. In the presence of a constant complexation buffer (CCB), the addition of sodium chloride and sodium hydrogencarbonate causes an increase in the limit of detection of the potentiometric response. When the procedure was tested in osteoblast-like cell culture medium no electrode interferences or method interferences were observed by the presence of proteins, i.e. the total and ionised calcium in this medium can be measured by potentiometric means.
INTRODUCTION

The potentiometric determination of calcium using ion-selective electrodes (ISEs) enjoys one special advantage over other methods of determining calcium (e.g. atomic absorption spectrometry or flame emission) which is the ability to monitor separately the ionised and total calcium concentration in a specific environment. By means of potentiometric measurements, it is feasible to access either the ionised calcium and determination of total calcium concentration, depending on the system undergoing investigation. In the field of biomedical research both determinations are quite useful considering that calcium plays an important role in the mineralisation process which takes place at the surface of biomaterials used for hard tissue replacement. Much has been done to understand the mineralisation process and its implications in orthopaedic, dental and cardiovascular surgery. When a biomaterial is exposed to extracellular matrix fluids a number of distinct processes occur immediately at the interface between the biomaterial (e.g. metal alloys, pure metals, ceramics or polymers) and extracellular fluids. These processes have not been fully elucidated as yet partly because they occur rapidly. Neither can they be studied in detail, via in vivo, since the methods of study are still imperfect and the technology available is far from being possible to monitor continuously the mineralisation process. However, the studies, via the use of in vitro models, can contribute to a better understanding of the surface interactions with the extracellular matrix and, by analogy, to have a clue of what happens in vivo. One of the parameters that needs to be controlled in the in vitro studies is the calcium concentration in the osteoblast-like cell culture medium. Osteoblast-like cells are the cells that
promote bone growth which are commonly used as cell culture test models to evaluate
the biocompatibility of materials used in medical devices. Calcium quantification in this
medium can be performed by using potentiometric procedures and calcium selective
electrodes.

As far as the calcium measurements is concerned, most of the work has dealt with the
determination of this species in aqueous media using the calibration plots procedure
based on the Nernst equation\textsuperscript{1-4}. Although these measurements use straightforward
procedures several limitations were found, namely how the interfering substances affect
the limit of detection and electrode potential drift. The use of a constant complexation
buffer (CCB) has partially eliminated these limitations and simultaneously improved the
accuracy of the experimental data attained by direct potentiometry procedures. The CCB
complexes calcium more strongly than the species originally present in the medium.

More recently, a few procedures for the determination of total calcium in blood
serum have been reported in the literature\textsuperscript{5-7}. These procedures were based on the pH
being adjusted to a value where calcium complexes are expected to dissociate (e.g.
pH=3.5), the use of a metallic cation such as Zn\textsuperscript{2+} to displace the binding calcium and
the use of the so called constant complexation buffer. From the reported results, it seems
that the use of CCB is the most appropriate procedure for total calcium determination in
physiological medium because it works at a pH value of 9.6 which avoids the
precipitation of inorganic compounds. Furthermore, this procedure minimises the
electrode drift. There is a main disadvantage, however, which consists in keeping the
ionic strength of the medium constant as required for the application of the Nernst equation.

The constancy of the ionic strength of the medium is a necessary condition for calcium determination using direct potentiometry procedures. Since our goal is to determine the total and free calcium by potentiometry in the osteoblast-like cell culture medium used to study in vitro the mineralisation process that occur at the biomaterials surface, the first step consists in obtaining the calibration curve under similar conditions. Considering the complex composition of the medium, several calibration plots were constructed, starting with a simple solution composition and subsequently adding the different components until reaching the desired medium composition. For all solutions investigated, four different values of ionic strength were used and the obtained results are discussed herein.

**MATERIALS AND METHODS**

*Cell Cultures*

Osteoblast-like cells were obtained from rabbit tibia bone marrow and their culture was performed according to the procedure reported in the literature.

Equipment and reagents

The experiments were performed under laboratory conditions using a Russell pH limited calcium selective electrode Model 93-3209 connected to a Crison MicroPH 2002 digital voltmeter. The external reference electrode was a Russell porous liquid junction Ag/AgCl/3 mol L\(^{-1}\) KCl. The pH was measured with a pH meter from Metrohm AG (CH-9100 Herisau, Switzerland).

The osteoblast-like cell culture medium consisted of \(\alpha\)-MEM (\(\alpha\)-Minimal Essential Medium Sigma M 0894) supplemented with 10% of antibiotic antimycotic solution 10 times concentrated (solution with 1000 units of penicillin per mL, 10 mg/mL of streptomycin and 25 mg/mL of amphotericin B), 10% foetal bovine serum, 10\(^{-8}\) mol/L of dexamethasone, 10 mmol/L of \(\beta\)-glycerophosphate and 2.52\(\times\)10\(^{-4}\) mol/L of ascorbic acid. All aqueous electrolyte solutions were made from deionised and distilled water and all reagents (purchased from Sigma) were of analytical grade.

Total calcium

Three series of five standard calcium solutions with concentrations in the range of 10\(^{-6}\)-10\(^{-2}\) mol/L were prepared by dilution of a 1.00 mol/L stock calcium chloride solution. The composition of the first set of standard calcium solutions was 1.00\(\times\)10\(^{-2}\) mol/L of iminodiacetic acid plus 5.00\(\times\)10\(^{-2}\) mol/L of ammonium chloride and concentrated ammonia solution up to pH=9.6 (ca. 8.8 mL/L). The second set of standard
calcium solutions has an identical composition of the previous one plus $1.32 \times 10^{-2}$ mol/L of sodium chloride and $2.60 \times 10^{-3}$ mol/L of sodium hydrogencarbonate. The third set of standard calcium solutions has the same composition of the set two plus 0.400 g/L of bovine serum albumin (>98%) and 1.00% of cell culture antibiotic antifungal solution 10 times concentrated. The last set simulates the osteoblast-like cell culture medium ten-fold diluted with deionised and distilled water. The dilution was made because it is under these conditions that the experimental measurements of total calcium in osteoblast-like cell culture medium will be carried out.

**Ionised calcium**

Variations of ionised calcium levels in osteoblast-like cell culture medium, during the mineralisation phase, were measured using calibration plots prepared with standards with composition similar to the culture medium two-fold diluted. The dilution was made because it is under these conditions that free calcium will be quantified in osteoblast-like cell culture medium. The composition of the standard calcium solutions, with calcium concentrations ranging from $10^{-6}$-$10^{-2}$ mol/L, was $6.62 \times 10^{-2}$ mol/L of sodium chloride, $1.31 \times 10^{-2}$ mol/L of sodium hydrogencarbonate, 2.00 g/L of bovine serum albumin, 8.50 mL/L of cell culture antibiotic antifungal solution 10 times concentrated and 10.0 mmol/L of Hapes buffer (pH=7.4).

In all standard calcium solutions, as well as in the samples, the ionic strength was adjusted to the desired value by adding increments of potassium chloride which is the recommended ionic strength adjustor for Russell's calcium selective electrodes. The
potential was monitored and the values of potential were recorded when the readings stabilised within $\pm 0.10$ mV for 5 min. It was observed that the calcium electrode has a fast response for concentrations above $10^{-4}$ mol/L but for lower concentrations the stability was attained only after 30 min. and the time increases as the concentration decreases.

To obtain accurate and reproducible results, no differences between standards and samples in ionic strength, pH, content of complexing agents and temperatures must exist$^9$. Also between measurements, the ion-selective electrode was kept in a solution of composition similar to that being analysed. The slope of the calibration plots obtained in this way usually does not change from day to day, but the electrode potential changes considerably because the $E^0$ depends on the lifetime of the electrode, the depth of immersion, etc., and therefore frequent recalibration is recommended$^{10-12}$.

**RESULTS AND DISCUSSION**

Calcium selective electrodes are based on the selectivity of the hydrophobic membrane phase for a certain cation. However its response is greatly influenced by other species (e.g. cations) present in the medium, causing a drift of the potentiometric response. Therefore, their workability and reproducibility is quite accurate for aqueous solutions but for physiological media and cell culture medium one has to investigate the most appropriate conditions, namely linearity zone, pH, and ionic strength influence.
In the present study, the ionic strength was calculated based on the following equation: \( I = \frac{1}{2} \sum_{i=1}^{n} C_i \times Z_i^2 \), where \( C_i \) is the concentration of the ions and \( Z_i \) is their charge, respectively.

The workability and reproducibility of the calcium selective electrode was checked by performing measurements in a simple aqueous solution with an ionic strength \( I = 0.05 \text{ mol/L} \). For this case a linear response was obtained (\( E/\text{mV} = 67.6 + 28.4 \times \log[Ca] \)), correlation coefficient (\( r \) =0.9997), giving a slope of 28.4 mV which is in good agreement with the theoretical value of 29.2 mV (at \( T = 294.15 \text{K} \)).

Since our goal was to measure the total calcium concentration in osteoblast-like cell culture medium which is quite complex, it was decided to investigate the ionic strength effect on the practical limit of detection, starting with a solution containing only the buffer and complexing agent after which the complexity of the solution was increased to a composition similar to the composition of the culture medium. Recording the potential of the electrode \textit{versus} pH in the presence of iminodiacetic acid it was observed that above pH of \( ca. 9.6 \) the potential remained constant indicating that calcium is completely complexed\(^7\). Bearing this in mind together with the fact that the conditional stability constant of calcium-iminodiacetate complex reaches its maximum between pH 9 and pH 13\(^7\), a pH value of 9.6 was choose to perform total calcium measurements. The effect of four different values of ionic strength, namely \( I = 0.16 \text{ mol/L}, I = 0.26 \text{ mol/L}, I = 0.36 \text{ mol/L} \) and \( I = 0.46 \text{ mol/L} \), for the solution containing \( 1.00 \times 10^{-2} \text{ mol/L iminodiacetic acid, 5.00 \times 10^{-2} mol/L of ammonium chloride and 8.80 mL/L of 25.0\%} \)
ammonia was studied and the results are illustrated in Fig. 1. It is observed that the limit of detection, defined as the concentration of calcium at the point of intersection of the extrapolated linear midrange and final low concentration level segments of the calibration plot\(^{13}\), decreases with a decrease of the ionic strength, \textit{i.e.} for \(I=0.16\) mol/L the limit of detection falls between \(10^{-6}-10^{-5}\) mol/L whereas for \(I=0.46\) mol/L the limit of detection is approximately \(10^{-4}\) mol/L. These values of the ionic strength were chosen bearing in mind that the value of the ionic strength of our physiological medium was \textit{ca.} 0.17 mol/L. Furthermore, it was experimentally observed that for higher values of ionic strength the drift in the potential is more accentuated.

\textbf{Figure 1-} Calibration plots in the presence of 1.00x10\(^2\) mol/L iminodiacetic acid, 5.00x10\(^2\) mol/L of NH\(_4\)Cl and 8.80 mL/L of 25.0\% NH\(_3\): (\(\ast\)) I=0.16 mol/L; (+) I=0.26 mol/L; (\(\Delta\)) I=0.36 mol/L and (o) I=0.46 mol/L.
For this set of experiments the slope in the linear region was found always to be higher than the Nernstian value, *i.e.* 29.2 mV (at 294.15 K). For instance, for I=0.46 mol/L the value found was 29.6 mV (in the linear range: E/mV=60.2+29.1xlog[Ca] and r=0.9990) whereas for I=0.16 mol/L the value found was 32.8 mV (E/mV=68.4+32.8xlog[Ca] and r=0.9999). This increase observed in the slope (when compared to the value measured for aqueous solution) is due to the equilibrium formed between the iminodiacetic acid and the calcium ion. The stability constant of the calcium complex, β, is 10^{2.6} which causes an approximately three-fold decrease of the calcium activity, but a nearly Nernstian working curve is still obtained\textsuperscript{14}.

In search for more evidence about the influence of the ionic strength on the electrode response, it was added to the previous solution 1.32x10^{-2} mol/L of sodium chloride and 2.60x10^{-3} mol/L of sodium hydrogen carbonate since these substances are present in the osteoblast-like cell culture medium. For the four ionic strengths studied, the achieved results indicate that the limit of detection increases for all values of I investigated. Similarly, the values of the slopes in the linear region increases as the ionic strength decreases. The increase of the limit of detection was expected since we have sodium ion present in the solutions which is an electrode/electrochemical interference\textsuperscript{13}. The Russell’s calcium electrode is twice as much sensitive to sodium as it is to potassium ion\textsuperscript{15} (*e.g.* a concentration of sodium of 2x10^{-2} mol/L cause the same error, *ca.* 10%, as 4x10^{-2} mol/L of potassium ion for a calcium concentration of 10^{-4} mol/L). On the other hand, the hydrogen carbonate anion usually reacts with calcium forming calcium hydrogen carbonate\textsuperscript{15} which works as a chemical interference\textsuperscript{13}. However it has been reported in the literature\textsuperscript{4,7} that the complexing agent, iminodiacetic acid, has a stronger

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affinity for calcium ion than hydrogencarbonate anion, avoiding the formation of calcium hydrogencarbonate.

The final stage consisted in adding the protein (albumin) and the antibiotic antimycotic solution to the solutions prepared for the previous measurements. These final solutions simulate the composition of the osteoblast-like cell culture medium. The achieved results are shown in Fig. 2 and clearly indicate that the last substances added do not cause any alteration in the potentiometric pattern, i.e. they are neither an electrode/electrochemical interference nor a chemical interference. This pattern agrees with what has been reported in the literature\textsuperscript{16} that in the presence of proteins, a small offset in the potentiometric response is seen at the first exposure to protein but no further alteration of the electrode response occurs. However the response time of the calcium electrode is increased after exposure to protein. Furthermore, it was found that the presence of 10\% (or lower) of organic matter did not affect the determination of calcium by the calcium selective electrode\textsuperscript{17}. Taking into consideration that during our experimental measurements the organic matter is approximately 1\% it will not interfere with the calcium response.
Figure 2- Calibration plots in simulated osteoblast-like cell culture medium ten-fold diluted for the determination of total calcium: (*) I=0.16 mol/L; (+) I=0.26 mol/L; (Δ) I=0.36 mol/L and (o) I=0.46 mol/L.

The effect of compounds such as sodium chloride, sodium hydrogen carbonate, cell culture antibiotic antimycotic solution and bovine serum albumin, and the effect of ionic strength on the limit of detection of ionised calcium was also studied. The same behaviour was observed when compared with what occurs with the total calcium determination, i.e. the detection limit decreases as ionic strength decreases. The influence of the ionic strength in ionised calcium quantification was also tested by variations of the potassium chloride content (ionic strength adjustor) in a solution which simulates the osteoblast-like cell cultures medium containing 10^{-4} mol/L of calcium. As
it can be seen in Fig. 3, an increment of *ca.* 0.26 mol/L in the ionic strength promoted a
decrease of 9.7 mV in the potential value read by the electrode, while the pH of the
solution was kept constant (pH=7.4). It can also be observed that between
I=0.35 mol/L and I=0.50 mol/L the addition of more potassium chloride had no effect in
the value of the calcium activity given by the ion-selective electrode. Several
explanations can account for the differences observed, namely the change in calcium
activity coefficient with the ionic strength (as the ionic strength increases the calcium
activity coefficient decreases) and the balance between free and bound ionised calcium
with protein.

**Figure 3**- Plot of potential vs. ionic strength of a solution containing $10^{-4}$ mol/L CaCl$_2$ and
simulating osteoblast-like cell culture medium two-fold diluted for the
determination of ionised calcium.
Calibration curves in the range of $10^{-4}$-$10^{-3}$ mol/L of calcium were used to quantify ionised and total calcium in several samples of osteoblast-like cell culture medium. The results are presented in Table 1 and show that during the mineralisation process there is a depletion of total calcium in the medium. This is in agreement with the results attained by microscopic observation of the cell cultures evolution and by histochemical assays, which evaluate mineralisation process by calcium deposits (alizarin red assay). Morphological observation of cell cultures (Fig. 4) showed that as the time of culture incubation increases, extracellular matrix mineralisation occurs and, on day 21 (Fig. 4B) mineralised nodules, evenly distributed through the cell culture, can be microscopically detected.

**Table 1** - Total and ionised calcium results obtained by potentiometry for osteoblast-like cell culture medium samples.

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<thead>
<tr>
<th>Sample</th>
<th>Total calcium (mol/L)</th>
<th>Ionised calcium (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$1.62 \times 10^{-3}$</td>
<td>$1.03 \times 10^{-3}$</td>
</tr>
<tr>
<td>2</td>
<td>$1.55 \times 10^{-3}$</td>
<td>$1.39 \times 10^{-3}$</td>
</tr>
<tr>
<td>3</td>
<td>$1.26 \times 10^{-3}$</td>
<td>$1.09 \times 10^{-3}$</td>
</tr>
<tr>
<td>4</td>
<td>$1.18 \times 10^{-3}$</td>
<td>$9.60 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Figure 4: Osteoblast-like cell cultures obtained from rabbit bone marrow showing: (A) absence of mineralisation at the 5th day of culture (X100) and (B) occurrence of mineralisation on day 21 (X100).
CONCLUSIONS

The analysis of the influence of the ionic strength on the calcium selective electrode potentiometric response indicates that the detection limit decreases as the ionic strength decreases. Also, for the determination of total calcium, the slope of the linear region deviates from the theoretical value and this deviation increases as the ionic strength decreases. This latest event is a function of the calcium complex stability constant with the iminodiacetic acid. Another conclusion was reached regarding the effect of the organic matter, which was that it does not influence the ionised and total calcium determination using the calcium selective electrode for the concentrations used: 2.00 g/L and 0.400 g/L of albumin, respectively.

From the practical point of view, the attained results points to the fact that lower values of ionic strength are more suitable for potentiometric calcium determinations. The value of the ionic strength chosen was 0.17 mol/L, which corresponds approximately to the value of the ionic strength of osteoblast-like cell culture medium (in order to keep the equilibrium of the system unchangeable). Furthermore, the calcium electrode herein used can be employed to measure the ionised and total calcium contents in osteoblast-like cell culture medium, and consequently is suitable to monitor the in vitro mineralisation process. Also, when mineralisation occurs the levels of total calcium in the medium decreases.
REFERENCES


II- CHAPTER 3

3- VOLTAMMETRIC AND SPECTROSCOPIC CHARACTERISATION OF CELL CULTURE MEDIUM PRE-TREATED FOR METAL IONS QUANTIFICATION
3.1- *In Vitro* Biomineralisation by Osteoblast-Like Cells:
Part II- Characterisation of Cellular Culture Supernatant

**ABSTRACT**

The quantification of total calcium, phosphorus, iron, chromium and nickel in cell culture medium by electrochemical or spectroscopic means may require digestion of samples. Nevertheless, when pH adjustment is performed for values higher than ca. 6.5, the formation of two phases occurs: a white precipitate and a limpid solution being observed. Analysing both phases using microelectrodes, atomic absorption spectrometry (AAS), diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, X-ray dispersive (XRD) analysis, scanning electron microscopy (SEM) and energy dispersive spectroscopic (EDS) analysis, it was observed that iron, chromium and nickel are not co-precipitating with the white solid phase. Also, if quantification of calcium, phosphorus and magnesium is intended a ten-fold dilution, at least, must be performed to avoid that most of these elements go into the precipitate. This knowledge is crucial if a mineralisation study is going to be made.
INTRODUCTION

Recent studies performed in vivo\textsuperscript{1,2,3} and in vitro\textsuperscript{4,5} have shown deleterious effects of AISI 316L stainless steel corrosion products obtained by electrochemical means. Their injection in mice caused not only ultrastructural changes in male reproductive organs\textsuperscript{1} and in liver parenchyme\textsuperscript{2}, but also accumulation of multinucleated giant cells and depletion of lymphocyte in spleen\textsuperscript{3}; previous in vitro studies suggested that such corrosion products inhibit the functional activity of human immune cells\textsuperscript{4} and skin fibroblasts\textsuperscript{5}. To evaluate the mineralisation process which occurs at the AISI 316L stainless steel biomaterial-bone interface, a number of in vitro tests were envisaged at our laboratory using osteoblast-like cell cultures\textsuperscript{6,7}. Several concentrations of the metal ions were added to the multi-well culture plates as described previously\textsuperscript{8} in order to assess the effects of metal ions released from this metallic biomaterial on the proliferation and function of rabbit osteoblast-like cells. The evidence has shown that the metal salts decrease the expression of the osteoblast phenotype, namely, alteration of the levels and temporal expression of alkaline phosphatase and retardation of the tissue mineralisation process. Stainless steel corrosion products showed a similar trend, although at a much lower extent.

We recently developed procedures to determine the amount of metal ions (e.g. Fe, Cr and Ni) in the cell culture medium using a mercury microelectrode (MFM) coupled with the adsorptive stripping voltammetry technique\textsuperscript{9,10,11,12}, instead of using AAS. However, the electrochemical metal ions quantification requires the use of an
appropriate ligand and adjustment of pH to the optimal region. Depending on the pH range (e.g. if it is higher than 6.5) formation of two distinct phases is observed: a white precipitate and a transparent liquid solution. The problem arises with the formation of the precipitate which may contain the metal ions to be quantified, leading to a default on the electrochemical response (considering that the peak current is proportional to the metal ion concentration). Thus, interest in analysing both phases by different spectroscopic techniques has arise.

In the present study, the characterisation of the solid and liquid phases formed from the digested osteoblast-like cell culture supernatants is discussed.

**MATERIALS AND METHODS**

Osteoblast-like cells were cultured in normal conditions and in the presence of a prepared metallic slurry of AISI 316L stainless steel according to the procedure described in Part 1. The composition of the osteoblast-like cell culture medium was previously mentioned (Part 1) but, it may be of interest to refer here that α-MEM contains essentially inorganic salts: calcium chloride; magnesium sulphate; potassium chloride; sodium chloride; sodium phosphate monobasic and sodium bicarbonate; twenty one amino acids and eleven vitamins. Samples of culture medium were collected and stored at -20°C for further quantification of free and total calcium (by potentiometry), inorganic and total phosphorus (colorimetric measurements), total iron, chromium and nickel (by voltammetry using microelectrodes). As previously
reported in the literature\textsuperscript{13,14}, the determination of free and total calcium by potentiometry using an ion-selective electrode\textsuperscript{13} and evaluation of inorganic phosphorus by colorimetry\textsuperscript{14} can be made directly on the samples without destruction of organic matter. In contrast, all the other quantifications required oxidative acid pre-treatment of the osteoblast-like cell culture medium even if traditional methods as colorimetry and AAS are used. Herein, digestion of samples was performed in a laboratory microwave oven (CEM Model MDS-2000) which has the ability to treat 12 samples simultaneously when placed in Advanced Composite vessels of 100 mL capacity. The maximum operating pressure of vessels is 1.4 MPa which allows to attain a temperature of \textit{ca.} 200°C. Digestion of 2 mL of the sample with the addition of 10 mL of suprapure nitric acid 65\% per vessel was carried out following the programme presented in Table 1. Because of the rate of pressure increase in the decomposition vessel, four programming stages were required to increase the pressure (temperature) during digestion and to reach and maintain the maximum pressure of 1050 KPa during a period of 30 min. This amount of time was chosen since, as verified by electrochemical measurements, the quantity of organic residues in the solution tend to be constant even if a longer decomposition time was used. After the digestion period, the decomposed solution was evaporated to dryness and the residues were dissolved in a volume of deionised and triply distilled water equal to the initial volume of the sample.
Table 1- Operational parameters for microwave oven digestion of samples

<table>
<thead>
<tr>
<th>Stage</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power (%)</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Pressure (KPa)</td>
<td>280</td>
<td>420</td>
<td>665</td>
<td>1050</td>
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<tr>
<td>TAP (min*)</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

*Time at the maximum pressure of the corresponding stage

The electrochemical studies of the culture medium using the square-wave voltammetry technique were performed with an AUTOLAB potentiostat/galvanostat Model PSTAT 10 coupled with an ECD Module (from Eco Chemie), a working mercury film microelectrode (MFM), an Ag/AgCl reference electrode and a cylindrical glassy carbon as counter electrode. The mercury microelectrode was prepared by electrodeposition of a mercury film onto a gold microdisk (radius=12.5 μm) by the application of a constant potential of 0.00 V\textsuperscript{15} vs. Ag/AgCl during a deposition time of 60 s.

Comparative measurements of the content of metal ions in osteoblast-like cell culture medium were made by the technique of electrothermal AAS with a Zeeman Atomic Absorption Spectrometer Model 4100 ZL from Perkin Elmer in conjunction with a graphite tube furnace.
DRIFT spectra were recorded using a Nicolet FT-510P spectrometer, equipped with a Collector device (Spectra-Tech). Spectra were obtained from powdered samples (water of the culture medium was evaporated under controlled atmosphere at a temperature of 60°C till complete dryness) approx. 3 mg sample/100 mg KBr (Uvasol, Merck), over the 4800-400 cm\(^{-1}\) wavenumber range, with a resolution of 2 cm\(^{-1}\). DRIFT spectra of the pure materials was run for comparison purposes whenever possible.

Elemental analysis for C, H, N, and S was carried out using a Carlo Erba E.A.-1108 elemental analyser. Up to 10 mg solid samples were analysed using appropriate clean tin containers without pre-treatment.

XRD profiles were collected in the 2θ range of interest, using a Philips semi-automatic X-ray diffractometer equipped with a graphite monochromator and a Cu target.

SEM analysis of the phases was carried out using a JEOL JSM-35 C microscope, and microanalysis performed with associated JEOL JSM-35 plus TRATOR TN-2000 systems for EDS analysis.
RESULTS AND DISCUSSION

Sample preparation according to the procedure described above lead to a colourless and limpid solution which pH must be adjusted to *ca.* 6.0 for chromium, *ca.* 8.0 for iron determination and to *ca.* 9.2 for nickel determination using a MFM in combination with a specific complexing agent: diethylenetriaminepentaacetic acid, catechol and dimethylgluoxime, respectively, for each metal ion. However, the increase of the pH solution for values higher than *ca.* 6.5 led to the formation of two distinct phases: one white precipitate (WP) insoluble in water, and one soluble phase. It was also observed that performing, at least, a ten-fold dilution the appearance of the WP was avoided. As the metal content in culture medium is very low, in the parts per billion range, this dilution can not be made in all samples without the risk of going out of the detection range of the mercury microelectrode and AAS techniques. Bearing in mind that the total content of iron, chromium and nickel aimed to be determined by electrochemical means, the WP and the soluble phase were analysed and characterised by several analytical and spectroscopic techniques to observe if they contain these metal ions. Since the optimal pH of chromium determination is *ca.* 6.0 (which is quite close of 6.5, pH of WP formation) possible losses of this ion by co-precipitation were also evaluated. It should be noted that, since the determination of phosphorus\(^{14}\) is carried out at very acidic pH, its quantification is not affected since in these conditions the WP is not formed.
Soluble phase

Square-wave voltammetry investigations in the soluble phase indicated the presence of an interference signal that appeared around -0.60 V (Fig. 1). The voltammetric elemental analysis of all the biological samples containing protein led to an identical signal\(^{17}\). This peak was attributed principally to the nitrobenzoic acids formed exclusively from two amino acids, phenylalanine and tryptophan, during the digestion process\(^{18}\). None of the other substances present in the biological materials made any appreciable contribution to the interference signal, as they are either completely degraded or present in such low concentrations that the interference signal of their reaction products are not detectable\(^{17}\). Other substances, such as methionine and linoleic acid, yield products which are voltammetrically inactive in the potential range studied\(^{17}\). So, it can be concluded that digestion under the conditions of a conventional pressure decomposition with nitric acid leads, even under optimised conditions, to the formation of residues of organic compounds that may interfere in the electrochemical quantification of metal ions in cell culture medium. To eliminate the interference signal, the solution had to be evaporated to fumes with perchloric acid\(^{19}\) (Fig. 1) increasing, however, the time of sample pre-treatment. Bearing this in mind, together with the fact that overlapping between the interference peak and the metal ions studied only occurs for iron, this additional step was only performed when iron was quantified. The reproducibility of the digestion procedure was also tested using several samples indicating that the intensity of the interference peak varied ca. 15\% which is tolerable within the experimental error of the digestion process.
Figure 1- Square-wave voltamnograms of the digested solution of osteoblast-like cell culture medium showing: (→) the nickel peak (A) and the presence of the interference signal (B); the nickel peak and (----) the absence of the interference signal due to evaporation with perchloric acid after the digestion period.

The DRIFT spectra of solid samples containing each of the phases in KBr are presented in Fig. 2. The spectrum corresponding to the soluble phase (Fig. 2a) is in agreement with the expected for a mixture of simple inorganic salts such as ammonium and nitrate\textsuperscript{20} which come from the digestion and adjustment procedures. Some contribution due to minor amounts of sulphate can also be ascertained. Elemental analysis (CHNS) of the dry residue of this phase is in line with our conclusions, the
Figure 2: Diffuse reflectance infrared Fourier transformed (DRIFT) spectra from (a) the dry residue of the soluble phase and (b) the white precipitate, obtained after adjustment to basic pH of the prepared sample.
following distribution of weights being found: N, 14.3; H, 1.8; S, 1.6 (% w). Sulphur derived not only from the amino acids but mainly from the magnesium sulphate salt existing in the cell culture medium. On the other hand no carbon was found suggesting complete destruction of the organic matter. From the DRIFT studies no evidence could be found for the presence of nitrobenzoic acid, detected by electrochemical means, possibly because the concentration of this compound in the sample is small in comparison to the other components. The XRD patterns of these samples (showing a strong triplet at 3.030 Å, 2.796 Å and 2.732 Å, with harmonic lines at 1.517 Å, 1.400 Å and 1.370 Å) are in agreement with the presence of different phosphates like, Ca₂Mg(PO₄)₂·2H₂O, Ca₅(PO₄)₃(OH), Mg₃(PO₄)₂, and Ca₄P₄O₁₁ together with NaNO₃.

The morphology of the dry residue of the soluble phase (Fig. 3a) has the appearance of a smooth cubic pellet encrusted with small crystals (Fig. 3b) fairly distributed by its surface. Semi-quantitative EDS analysis (Figs. 4a-b) shows that the pellet is mainly composed by sodium, while the crystals have in their composition high contents of sulphur, calcium, potassium and chloride, but practically no phosphorus and magnesium (Table 2). The balance between the cation and anion contents it is almost equivalent, slightly in favour of the positive charged ones.
Figure 3- Scanning electron micrographs (SEM) of the dry residue of the soluble phase obtained after adjustment to basic pH of the solution resulting from the digestion procedure. (a) General view (original magnification X60); (b) detail of one pellet (original magnification X650).
Figure 4- Energy dispersive spectra (EDS) from the matrix of the pellet (a) and from the encrusted crystallites (b), observed upon analysis of the dry residue of the soluble phase obtained after adjustment to basic pH of the digested sample.
Table 2- SEM/EDS analysis of the different phases formed upon increase of the pH of the culture medium (atom %)

<table>
<thead>
<tr>
<th>Element</th>
<th>Soluble Phase</th>
<th></th>
<th>White Precipitate</th>
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<tbody>
<tr>
<td></td>
<td>Matrix</td>
<td>Crystal</td>
<td>Matrix</td>
<td>Crystal</td>
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<tr>
<td>Na</td>
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<td>9.52</td>
<td>30.37</td>
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<tr>
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<td>0.15</td>
<td>3.39</td>
<td>---</td>
</tr>
<tr>
<td>P</td>
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<td>0.79</td>
<td>4.43</td>
<td>31.64</td>
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<tr>
<td>Cl</td>
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<td>9.75</td>
<td>58.24</td>
<td>7.28</td>
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<tr>
<td>K</td>
<td>1.87</td>
<td>13.69</td>
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<td>1.66</td>
<td>---</td>
</tr>
<tr>
<td>Ca</td>
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<td>6.26</td>
<td>46.79</td>
<td>---</td>
</tr>
<tr>
<td>S</td>
<td>0.00</td>
<td>37.33</td>
<td>0.19</td>
<td>0.58</td>
<td>---</td>
</tr>
</tbody>
</table>

**White precipitate**

Possible losses of metal ions due to adsorption and/or co-precipitation with the WP were evaluated by comparison of the quantitative results obtained by electrochemical measurements using MFM (at near neutral, pH 6.0, or at basic pH where formation of WP is observed) and by AAS (at acidic pH *i.e.* precipitation did not occurred). Good agreement was found indicating that the WP did not promote the decrease of the content of the metal ions studied (Fe, Cr and Ni) in the solution$^{9,11-12}$.
The DRIFT spectrum of the WP (Fig. 2b) is consistent with the formation of a tribasic phosphate\textsuperscript{20,21}. Our assignment was substantiated with basis on the comparison with a pure spectrum of Ca$_3$(PO$_4$)$_2$. The resulting spectrum (not shown) gave a perfect match with respect to peak position within the 500 to 1020 cm$^{-1}$ region, but with some difference in band intensity and broadening, indicating that something is interfering. One possible explanation for the observed interference could lie in the presence of nitrate salts which show some bands at the expected wavenumbers\textsuperscript{21}, including those in the 1350 to 1400 cm$^{-1}$ range. Part of broadening and band shift in frequency values can result from salt mixture (\textit{e.g.} a NaNO$_3$ / KNO$_3$ mixture), which was confirmed by combining the spectra of the pure salts in different proportions. The remaining part of the spectrum is due to the (OH) group\textsuperscript{21}, which is a good indicator for a basic calcium phosphate such as Ca$_5$(PO$_4$)$_3$(OH). Apatite composites are known to display modified spectra with relation to the true hydroxyapatite standard\textsuperscript{22}.

Comparing the spectra in Fig. 2 (a and b), there is a clear evidence for the existence of a common species in both phase, which is the major component of the soluble phase as described in the previous section. Elemental analysis of the WP shows minor amounts of carbon (found: N, 6.6; C, 0.6; H, 1.1 \% w) which may be due to the nitrobenzoic acids formed during the digestion procedure. In contrast to the soluble phase no sulphur was found, which is in agreement with the high solubility of sulphate salts at different pH's.
The DRIFT results are in line with the qualitative analysis of the XRD patterns, where a triplet (3.035 Å, 2.818 Å and 2.738 Å) consistent with a compound like Ca$_2$Mg(PO$_4$)$_2$:2H$_2$O was identified. However, the patterns are also very consistent with the presence of Ca$_5$(PO$_4$)$_3$(OH) and NaNO$_3$. The phosphate complexes are extremely insoluble, principally in neutral or basic solutions, (e.g. Ca$_3$(PO$_4$)$_2$ shows a $K_{sp}$ of $2.83 \times 10^{-30}$ at 37°C$^{23}$ which is in agreement with a compound of this type being the major component of the WP phase. SEM of the WP deposit shows the formation of aggregates like grains from various shapes (Fig. 5a) with irregular surface, covered with small crystallites (Fig. 5b). Analysis of the surface by semi-quantitative EDS (Figs. 6a-b) shows that the matrix of the structure (which means the space between the crystallites) is mainly composed by sodium and chloride, while the crystallites do present magnesium, phosphorus and calcium in an atomic ratio of 2:3 (Table 2), as expected for a compound like Ca$_3$(PO$_4$)$_2$. Some other elements could also be found in the constitution of this solid phase although in minor amounts.
Figure 5- Scanning electron micrographs (SEM) of the white precipitate formed after basification of the colourless and limpid solution obtained from the digestion procedure. (a) General view of the white solid (original magnification X55); (b) detail of one grain covered with crystallites (original magnification X360).
Figure 6- Energy dispersive spectra (EDS) of the matrix of the grain (a), and of a crystallite (b), observed upon analysis of the white precipitate formed after basification of the colourless and limpid solution obtained from the digestion procedure.
CONCLUSIONS

Characterisation of osteoblast-like cell culture medium after pressure digestion was performed by electrochemical means and spectroscopic techniques. It is shown by the example of the determination of nickel by square-wave voltammetry using a MFM that an interference signal exists around -0.60 V (vs. Ag/AgCl) caused by the reduction of nitrobenzoic acids at the mercury film surface. Nitrobenzoic acids arise from the decomposition of two amino acids, phenylalanine and tryptophan, during digestion and consequently this interference will be present in the voltammetric analysis of all biological samples. For some metal trace quantification, complete destruction of the organic matter is necessary, which requires to attack the sample with perchloric acid\textsuperscript{19}.

The characterisation of the two phases formed during pH adjustment of the digested osteoblast-like cell culture medium showed that one must be careful if quantification of calcium, phosphorus and magnesium is made at near neutral or basic pH. A proper dilution of, at least, ten times must be done to avoid that most of these elements go into the precipitate. The knowledge of this fact is crucial if the biomineralisation, \textit{i.e., the biologically-controlled} processes characterised by the cellular control of mineral formation\textsuperscript{24}, is to be studied since mineral (hydroxyapatite) deposition must requires consumption of calcium and phosphate ions from the culture medium. Consequently, monitoring the variation of concentration of total and ionised calcium and phosphorus in the cellular culture supernatant, throughout the culture period, is regarded as a measure of the biomineralisation process occurring in cell cultures.
Results obtained in the present study indicate that the normal behaviour of osteoblastic cells as well as the mineralisation process may be impaired by the presence of metal ions which may help to explain the pathological bone\textsuperscript{25,26,27} and bone loss\textsuperscript{25,28,29} reported to occur surrounding some metal implants. These considerations suggest the importance of investigating the changes in concentration of the metal ions, and not only of calcium and phosphorus. In vitro osteoblast-like cell cultures, used to study the bone-implant interface, constitute ideal systems to evaluate the uptake of (and of which) metal ions from the incubation medium by cells and correlate it with the disturbance of the proliferation and function of osteoblast-like cells (the mineralisation process is one specific function of osteoblasts). The combination of a MFM with square-wave voltammetry provides excellent sensitivity and gives reliability to determine metal ions in osteoblast-like cell culture medium, after appropriate pressure digestion, constituting a suitable and useful alternative to the traditional method of AAS due to the short analysis time and it is a non-destructive technique.

**REFERENCES**


II- CHAPTER 4

4- DOSE- AND TIME-EFFECTS OF 316L STAINLESS STEEL CORROSION PRODUCTS IN IN VITRO BONE FORMATION AND OSTEOBLASTIC BEHAVIOUR
4.1- Effects of AISI 316L Corrosion Products in *in Vitro* Bone Formation

**Abstract**

Rat bone marrow cells were cultured in experimental conditions that favour the proliferation and differentiation of osteoblastic cells (i.e., \(2.52 \times 10^4\) mol L\(^{-1}\) ascorbic acid, \(1.00 \times 10^{-3}\) mol L\(^{-1}\) β-glycerophosphate and \(10^{-8}\) mol L\(^{-1}\) dexamethasone) in the absence and in the presence of stainless steel corrosion products, for a period of 18 days. An AISI 316L stainless steel slurry (SS) was obtained by electrochemical means and the concentrations of the major metal ions, determined by atomic absorption spectrometry, were \(8.78 \times 10^{-3}\) mol/L of Fe, \(4.31 \times 10^{-3}\) mol/L of Cr and \(2.56 \times 10^{-3}\) mol/L of Ni. Bone marrow cells were exposed to \(1.00 \times 10^{-2}\%\), \(1.00 \times 10^{-1}\%\) and \(1.00\%\) of the SS and at the end of the incubation period, control and treated cultures were evaluated by histochemical assays for the identification of the presence of alkaline phosphatase positive cells but also calcium and phosphate deposition. Cultures were further observed by scanning electron microscopy. Levels of total and ionised calcium and phosphorus in the culture media collected from control and metal exposed cell cultures were also quantified.

Results showed that control cultures presented high amounts of alkaline phosphatase positive cells and exhibited formation of calcium and phosphate deposits. The presence of \(1.00 \times 10^{-2}\%\) SS caused no detectable biological effects in these cultures, \(1.00 \times 10^{-1}\%\) SS impaired osteoblastic behaviour and, \(1.00\%\) SS resulted in cell death. In the absence of bone cells, levels of total, and ionised calcium and phosphorus in the control and metal added culture medium were similar throughout the incubation period. A significant decrease on the levels of ionised calcium and phosphorus were observed in the culture medium of control cultures and also in cultures exposed to \(1.00 \times 10^{-2}\%\) SS after two weeks of incubation, an event related with the formation of mineral deposits in these cultures. In cultures grown in the presence of \(1.00 \times 10^{-1}\%\) and \(1.00\%\) SS corrosion products, levels of calcium and phosphorus were similar to those observed in the absence of cells.

Results showed that stainless steel corrosion products above certain concentrations may disturb the normal behaviour of osteoblast-like rat bone marrow cell cultures.
INTRODUCTION

The cellular events taking place at the bone/material interface can be determinant for the success of the implant long-term performance\textsuperscript{1-4}. There is some concern over the use of metallic biomaterials in orthopaedic surgery, based on the release of implant debris and metallic ions in tissues adjacent to implanted materials and their distribution throughout the body via systemic circulation\textsuperscript{5-6}. Several studies have demonstrated high metal concentrations in body fluids (including serum and urine) of patients with knee and hip prosthesis and other authors have further suggested a potential role for ion and particles released from metal implants in various pathological bone and tissue conditions\textsuperscript{7-12}.

Well characterised osteoblast-like cell cultures have been used as a suitable \textit{in vitro} model to study the interactions of biomaterials and their degradation products with bone cells at the tissue-implant interface\textsuperscript{13-16}. Previous work performed in osteogenic cell cultures suggest acute and long-term effects of metal ions, found in commonly used orthopaedic implants, on the proliferation and function of osteoblast lineage cells\textsuperscript{17-21}.

AISI 316L stainless steel corrosion products obtained by electrochemical means have been a matter of intense research at this laboratory. Both \textit{in vitro} and \textit{in vivo} studies have shown deleterious effects of stainless steel corrosion products in several organs and tissues\textsuperscript{21-26}. In this work, rat bone marrow cultures obtained in experimental conditions that favour the proliferation and differentiation of osteoblastic cells were
cultured in the presence of AISI 316L stainless steel corrosion products at different concentrations, for a period of 18 days. Control and treated cultures were evaluated by histochemical assays for the identification of the presence of alkaline phosphatase and calcium and phosphates deposition and examined by scanning electron microscopy (SEM); levels of total and ionised calcium and phosphorus in the culture media collected from control and metal added cultures during the incubation period, were also quantified. The concentrations of the major metal ions present in the SS were in the range of iron, chromium and nickel levels found in tissues adjacent to stainless steel implants.  

**MATERIALS AND METHODS**

*Metallic slurries*

A metallic AISI 316L stainless steel slurry was prepared by anodic dissolution in Hank's Balanced Salt Solution (HBSS) and the resulting concentrations of the major metal ions were determined by atomic absorption spectrometry (AAS): \(8.78 \times 10^{-3}\) mol/L of Fe, \(4.31 \times 10^{-3}\) mol/L of Cr and \(2.56 \times 10^{-3}\) mol/L of Ni. After pH adjustment (to 7.4) and sterilisation in an autoclave, further solutions were obtained by successive dilutions.
Cell culture

A primary culture of osteoblast-like cells was established using a young adult male Wistar rat bone marrow suspension obtained accordingly with the method described by Maniotopoulos et al. Briefly, the femora were excised aseptically, cleaned of soft tissues, and passed through 2 washes, 10 min. each, of α-MEM (α-Minimum Essential Medium, SIGMA M 0894) containing antibiotic antimycotic solution (SIGMA A 9909, 1700 units of penicillin per mL, 1.7 mg/mL of streptomycin and 4.2 μg/mL of amphotericin B). Then, the epiphyses were removed, and the marrow flushed out using a 10 mL syringe with a 20 G needle and containing culture medium. The cell suspension obtained was distributed, after homogenisation, through three 50 mL culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Medium was changed 24 hours later to remove non-adherent cells. The culture medium consisted of α-MEM supplemented with 10% foetal bovine serum, antibiotic antimycotic solution (170 units of penicillin per mL, 0.17 mg/mL of streptomycin and 0.42 μg/mL of amphotericin B), 10⁻⁸ mol L⁻¹ dexamethasone, 2.52x10⁻⁴ mol L⁻¹ ascorbic acid and 1.00x10⁻² mol L⁻¹ β-glycerophosphate and was changed twice a week.

Cells of the first subculture (obtained by trypsination of cells 7 days after the beginning of the incubation) were seeded at a concentration of 10⁴ cells/cm² in 35 mm diameter tissue culture dishes and cultured in normal culture medium (that used in the primary culture) and in the presence of 1.00x10⁻²%, 1.00x10⁻¹% and 1.00% of the stainless steel slurry for a period of 18 days. For control quantification experiments, 35 mm cell free tissue culture dishes containing culture medium with and without added
stainless steel corrosion products were incubated and treated as the cell culture plates. The culture media were changed every 2 days, collected and analysed for quantification of total and ionised calcium and phosphorus. At the end of the incubation period (18 days) control and treated cultures were evaluated by histochemical assays for the identification of the presence of alkaline phosphatase and calcium and phosphates deposition and examined by SEM.

**Histological methods**

a) **Light microscopy**

The presence of ALP positive cells and phosphate or calcium deposits were visualised by histochemical staining. The cultures were fixed with 1.5% glutaraldehyde in 0.14 mol L⁻¹ sodium cacodylate buffer and rinsed with distilled water.

**ALP staining**

Fixed cultures were incubated during 1 hour in the dark with a mixture, prepared in Tris buffer pH=10, containing 2 mg/mL of Na-α-naphtyl phosphate (reacting substrate) and 2 mg/mL of fast blue RR salt. The incubation was stopped by rinsing the samples with water and then, the cells were observed in a Olympus BH-2 optical microscope. ALP positive cells stained brown.
Phosphates staining

Phosphates deposits were assessed by the von Kossa technique\textsuperscript{29} \textit{i.e.}, the fixed cultures were covered with a 1% silver nitrate solution and kept for 1 hour under UV light. After rinsing, a 5.0% sodium thiosulphate solution was added for 2 min. and cultures were washed again. Phosphate deposits stained black.

Calcium staining

The fixed cultures were covered with a 1.0% S alizarin sodium sulfonate solution (0.028% in NH\textsubscript{4}OH), pH=6.4, for 2 min. and then rinsed with water and acid ethanol (ethanol, 0.01% HCl)\textsuperscript{29}. Calcium deposits stained red.

b) Scanning electron microscopy

Cultures were fixed as for light microscopy and dehydrated in a graded ethanol series. Critical point drying of the samples was followed by gold sputtering. The preparations were examined in a Philips SEM 525M at 15 kV.

Ionised and total calcium and phosphorus quantifications

Ionised and total calcium

The free and total calcium contents in cell culture media were evaluated by potentiometric means accordingly with the procedure described elsewhere\textsuperscript{30}. The potential was monitored using a calcium selective electrode and an Ag/AgCl reference electrode, and the values of potential were recorded when the readings stabilised within
± 0.10 mV for 5 min. Calibration curves were prepared with standards with a composition similar to the culture medium two-fold or ten-fold diluted. These dilutions were made because it is under these conditions that free (a two-fold dilution) and total (a ten-fold dilution) calcium was quantified in osteoblast-like cell culture medium. The ionic strength of the standard calcium solutions as well as of the samples was adjusted and maintained (during the dilution of the samples) at ca. 0.17 mol/L which corresponds to the value of the ionic strength of culture medium in order to keep the equilibrium of the system unchangeable\textsuperscript{30}.

**Ionised and total phosphorus**

The inorganic (Pi) and total phosphorus (P) were determined colorimetrically using the Fiske and Subbarow procedure\textsuperscript{31}. For Pi quantification, the culture medium was treated with tricloroacetic acid to precipitate protein and lipid-bound phosphates. The supernatant fluid was reacted with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulfite, sodium sulfite and 1-aminonaphthol-4-sulfonic acid was then added to reduce the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the colour was measured at 660 nm in a Shimadzu UV-VIS spectrometer.

For P quantification, samples of cell culture medium were, first, digested in a microwave oven (CEM Model MDS-2000) with nitric acid (suprapure 65%) in order to destroy organic matter. Then, the solutions obtained were treated as for Pi quantification (obviously the tricloroacetic acid step was not performed). All values were calculated from standard curves read at the same wavelength.
All data presented correspond to the mean of, at least, three measurements of the same sample. Standard deviations were not represented in Fig. 4 (in the Results and Discussion section) to avoid superposition of data and symbols, however the minimum and maximum values for the coefficient of variation, calculate as the standard deviation divided by the mean of the set of data and multiplied by 100\(^32\), are represented in Table 1 (in the Results and Discussion section).

**RESULTS AND DISCUSSION**

Previous studies have shown that rat bone marrow cultures obtained in the experimental conditions described above present osteoblast features, namely, high alkaline phosphatase activity and formation of a mineralised extracellular matrix\(^{28,33-34}\). The presence of 2.52x10\(^{-4}\) mol L\(^{-1}\) ascorbic acid, 1.00x10\(^{-2}\) mol L\(^{-1}\) \(\beta\)-glycerophosphate and 10\(^{-8}\) mol L\(^{-1}\) dexamethasone favours the formation of cultures of osteoblast phenotype in several bone cell culture systems\(^{28,35-39}\).

In this work, rat bone marrow cells (first subculture) were cultured during 18 days in control culture medium and in the presence of 1.00x10\(^{-2}\)%, 1.00x10\(^{-1}\)% and 1.00% of the SS prepared as described in materials and methods. At the end of the incubation period, control and treated cultures were evaluated by histochemical assays and examined by SEM. Quantification of total and ionised calcium and phosphorus in the control and metal added culture media (incubated in the absence and in the presence of bone cells) during the 18 days culture period was also performed.
Histochemical assays

Bone marrow cultures grown in control conditions showed the formation of three-dimensional nodular structures that were opaque in appearance and increased in size with incubation time. Histochemical assays showed a strong positive reaction for the presence of ALP and also for calcium and phosphates deposition (Fig. 1a, e, i). Histochemical staining was mainly associated with the three-dimensional nodules. Bone marrow cells cultured in the presence of the lowest concentration of the stainless steel slurry tested (1.00x10^{-2}\% \text{, i.e.,} 8.78x10^{-7} \text{ mol/L of Fe, 4.31x10^{-7} \text{ mol/L of Cr and 2.56x10^{-7} mol/L of Ni}}) showed a similar behaviour (Fig. 1b, f, j). No marked differences were observed in the intensity of the histochemical reactions in this experimental situation, as compared with control cultures. In the presence of 1.00x10^{-1}\% of SS corrosion products, cultures exhibited a positive histochemical reaction for the presence of alkaline phosphatase (although weaker than that observed in control cultures) but calcium and phosphates deposition was not observed (Fig. 1c, g, k). The presence of 1.00\% SS slurry in the incubation medium (corresponding to 8.78x10^{-5} \text{ mol/L of Fe, 4.31x10^{-5} mol/L of Cr and 2.56x10^{-5} mol/L of Ni}) was toxic and resulted in cell death; only few cells with an altered morphology were observed in this situation (Fig. 1d, h, l).
Figure 1- Histochemical assays on 18 days rat bone marrow cultures (X100).

Cells grown in control conditions (a, e, i), or in the presence of $1.00 \times 10^{-2}\%$ (b, f, j), $1.00 \times 10^{-1}\%$ (c, g, k) and $1.00\%$ (d, h, l) SS corrosion products were assayed for ALP positive cells (a-d), calcium deposits (e-h) and phosphate deposits (i-l).
Figure 1 (continued)
Scanning electron microscopy

SEM observation of control and metal treated cultures confirmed the results observed on the histochemical assays. Control cultures showed the presence of cells and numerous globular deposits that seemed to fuse in regions of high density (Fig. 2). This aspect is in accordance to other previously reported studies in similar culture systems\textsuperscript{28,33-34}. Fig. 3 compares the appearance of control and treated cultures. In the presence of 1.00\times10^{-2}\% SS cultures presented globular deposits (Fig. 3c, d) similar to those observed in control cultures (Fig. 3a, b). In both cases, X-ray microanalysis of the globular deposits showed the presence of Ca and P peaks (results not shown). These formations were absent in bone marrow cultures exposed to 1.00\times10^{-1}\% SS, where only cells and a network of collagen fibbers could be observed (Fig. 3e, f). Cultures treated with 1.00\% SS showed evident signs of deterioration as compared to control cultures (Fig. 3g).
Figure 2- SEM appearance of rat bone marrow cultures grown in control conditions for 18 days. Bar=10 μm.
Figure 3- SEM appearance of selected areas of control and metal added 18 days rat bone marrow cultures. Cells grown in control conditions (a, b) and in the presence of $1.00 \times 10^{-2}\%$ (c, d), $1.00 \times 10^{-3}\%$ (e, f) and $1.00\%$ (g) SS corrosion products. a, c, e, g-Bar=0.1 mm; b, d, f-Bar=10 μm.
Figure 3 (continued)
Total and ionised calcium and phosphorus in the culture media

Culture media from control and metal added cultures were collected every two days (and cultures refed with fresh medium) during the 18 days incubation period and analysed for total and ionised calcium and phosphorus concentration. Quantification of these species in control and treated culture media incubated in the absence of bone cells in the same experimental conditions as cell cultures, was also performed. Results are presented in Fig. 4 and Table 1. Levels measured of total and ionised calcium and phosphorus were not cumulative, as culture medium was totally replaced every 2 days; values shown reflect changes occurring in intervals of 2 days throughout the culture period.

In the absence of bone cells, levels of total and ionised calcium and phosphorus in control and metal treated culture media were similar throughout the 18 days of incubation. Total calcium concentration ranged from a minimum of $1.80 \pm 0.14$ mmol/L to a maximum of $2.50 \pm 0.060$ mmol/L and most of it was ionised ($1.24 \pm 0.014$ mmol/L to $1.70 \pm 0.040$ mmol/L); concentration of total phosphorus ranged from $9.00 \pm 0.045$ mmol/L to $12.0 \pm 0.10$ mmol/L and content of ionised phosphorus was significantly lower ($1.30 \pm 0.018$ mmol/L to $1.80 \pm 0.014$ mmol/L). Ionised calcium and phosphorus originate from calcium and phosphates compounds present in α-MEM (2.40 mmol/L and 0.900 mmol/L, respectively) and in foetal bovine serum (ca. 3.8 mmol/L and ca. 4.0 mmol/L, respectively). Addition of β-glycerophosphate (10.0 mmol/L) was responsible for the increased value observed for total phosphorus concentration, as compared to that found for ionised phosphorus. This ester phosphate
Figure 4—Levels of total and ionised calcium (a, b) and phosphorus (c, d) in the culture media from control and metal added cultures, collected every 2 days throughout the 18 days incubation period. Levels of these species in the normal and metal treated culture media incubated in the absence of cells is also shown. (𝒟) control culture medium; (●) culture medium from control cultures; (+) 1.00x10^{-2}\% SS culture medium; (×) culture medium from 1.00x10^{-2}\% SS cultures; (Δ) 1.00x10^{-1}\% SS culture medium; (▲) culture medium from 1.00x10^{-1}\% SS cultures; (Ο) 1.00\% SS culture medium; (●) culture medium from 1.00\% SS cultures.
Table 1- Minimum and maximum values for the coefficient of variation (%) of the levels of total and ionised calcium and phosphorus, measured in the culture media during the 18 days incubation period.

<table>
<thead>
<tr>
<th></th>
<th>Total calcium</th>
<th>Ionised calcium</th>
<th>Total phosphorus</th>
<th>Ionised phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control culture medium</td>
<td>1.2 - 6.6</td>
<td>0.69 - 6.0</td>
<td>0.30 - 3.0</td>
<td>0.00 - 3.8</td>
</tr>
<tr>
<td>Culture medium from control cultures</td>
<td>3.9 - 6.3</td>
<td>1.5 - 6.5</td>
<td>0.11 - 1.6</td>
<td>0.040 - 0.58</td>
</tr>
<tr>
<td>1.00x10⁻²% SS culture medium</td>
<td>1.2 - 7.0</td>
<td>0.68 - 6.2</td>
<td>0.30 - 2.1</td>
<td>0.00 - 4.1</td>
</tr>
<tr>
<td>Culture medium from 1.00x10⁻²% SS cultures</td>
<td>4.2 - 6.8</td>
<td>1.1 - 4.4</td>
<td>0.14 - 2.8</td>
<td>0.11 - 2.1</td>
</tr>
<tr>
<td>1.00x10⁻¹% SS culture medium</td>
<td>2.0 - 5.9</td>
<td>0.69 - 3.6</td>
<td>0.00 - 0.79</td>
<td>0.14 - 2.7</td>
</tr>
<tr>
<td>Culture medium from 1.00x10⁻¹% SS cultures</td>
<td>3.0 - 6.7</td>
<td>0.70 - 4.4</td>
<td>0.33 - 2.2</td>
<td>0.17 - 3.0</td>
</tr>
<tr>
<td>1.00% SS culture medium</td>
<td>0.80 - 5.6</td>
<td>0.70 - 5.7</td>
<td>0.27 - 1.7</td>
<td>0.14 - 3.8</td>
</tr>
<tr>
<td>Culture medium from 1.00% SS cultures</td>
<td>2.6 - 6.3</td>
<td>0.70 - 6.6</td>
<td>0.00 - 2.7</td>
<td>0.12 - 4.1</td>
</tr>
</tbody>
</table>

provides a potential source of phosphate ions required for the mineralisation process in bone nodules forming cultures (deposition of calcium phosphate)²⁸⁻⁴⁰⁻⁴². One can notice that calcium and phosphorus content in culture medium varied slightly during the time
course of the experiment. This fact may be explained bearing in mind that the culture medium has to be prepared several times during the assay and that is practically very difficult to pipette the culture medium uniformly due to the presence of proteins (which bind calcium and phosphorus). So, discrepancies may come basically from two steps: - during medium introduction in the culture dishes, and - during collection of aliquots for quantification. The possibility of the incorrect application of the selected methods may be discarded since good precision and accuracy were obtained in preliminary comparative studies\textsuperscript{30} and also, other authors\textsuperscript{41,43} reported similar variations in calcium and phosphorus content using other techniques.

Culture media collected from control cultures and also cultures exposed to 1.00x10\textsuperscript{-3}\% SS showed increasing concentrations of ionised phosphorus in the first two weeks of culture (maximal levels were about 8.0 to 9.5 mmol/L, compared with 1.3 to 1.8 mmol/L present in the absence of bone cells), although concentration of total phosphorus remained constant (Fig. 4). These results suggest that most of the β-glycerophosphate added is converted to inorganic phosphate, most probably by alkaline phosphatase, present in high levels, according to the results observed in the histochemical assays (Fig. 1). Ability of the cultures to hydrolyse this compound appeared to be maximal between 8 and 12 days of culture. Levels of total and ionised calcium in the culture media did not change significantly during this phase of the culture. However, after approximately two weeks of culture, levels of calcium and phosphorus (almost all ionised at this stage of the cultures) in the culture media decreased significantly. This consumption of ionised calcium and phosphorus (inorganic phosphate) is most probably related with the formation of mineral deposits (calcium
phosphates deposition), as previous work has shown that mineralisation of the extracellular matrix in this culture system (control conditions) is observed from two weeks onward\textsuperscript{28,33-34}.

In cultures grown in the presence of $1.00\times10^{-1}\%$ SS, part of the $\beta$-glycerophosphate added was hydrolysed and measured levels of inorganic phosphate in the incubation medium increased throughout the incubation period. Hydrolysis of this compound was probably performed by alkaline phosphatase, as this enzyme is present in these cultures (although, at lower levels than those observed in control conditions, Fig. 1) and it is known to have high efficacy in hydrolysing this ester phosphate; other phosphatases present in bone cells, \textit{i.e.}, inorganic pyrophosphatase, can also contribute to the hydrolysis of $\beta$-glycerophosphate. However, levels of total calcium and phosphorus in the incubation media were similar throughout the 18 days incubation period and close to those found in the absence of cells, contrasting with the significant decrease observed in control and $1.00\times10^{-2}\%$ SS cultures, after two weeks of incubation.

Cultures grown in the presence of $1.00\%$ SS did not show significant changes in the concentration of total and ionised calcium and phosphorus during the incubation period and levels were similar to those measured in the absence of cells. These results were expected as histochemical assays and SEM observation of the cultures have shown that the presence of SS at this concentration was too toxic and resulted in cell death.

Results presented in Fig. 4 show that values found for total calcium and phosphorus concentrations are, in majority, lower than the expected theoretical values based upon
the proportion of each compound in the culture medium (α-MEM + 10% foetal bovine serum + 10.0 mmol/L β-glycerophosphate) i.e., ca. 2.8 mmol/L for calcium and ca. 11.3 mmol/L for phosphorus suggesting that some constant precipitation occurs in all culture plates during the 18 days of incubation. However, this non-biological mineral deposition seems to be insignificant when compared with the high consumption of calcium and phosphorus observed during the biomineralisation that occurred in control and 1.00x10^{-2}% SS cultures from approximately two weeks onward (Fig. 4).

The behaviour observed in control cultures concerning levels of calcium and phosphorus in the culture medium during the culture period appears to be in agreement with that reported in the literature in studies performed in similar culture systems (cultures forming mineralised bone nodules in these experimental conditions)\textsuperscript{28,40-42}. These studies have shown that alkaline phosphatase and organic phosphate play a crucial role in the initiation of the mineralisation process. Studies performed in the presence and in the absence of the alkaline phosphatase inhibitor levamisole showed that β-glycerophosphate is utilised as a substrate for this enzyme and that it is rapidly converted to inorganic phosphate, required for the formation of calcium phosphate\textsuperscript{40-41}. In addition, it was also shown that incorporation of calcium in the cell culture occurs during the mineralisation process\textsuperscript{41-42}.

The results reported in this work suggest that evaluation of the concentration of total and ionised calcium and phosphorus in the incubation medium throughout the culture period may be regarded as a measure of the mineralisation process occurring in these cell cultures, as calcium phosphates deposition requires consumption of calcium and
phosphate ions from the culture medium. The results reported and presented in Figs. 1-4 showed that the presence of 1.00x10^{-2}\% \text{SS} (corresponding to 8.78x10^{-7} \text{mol/L of Fe, 4.31x10^{-7} mol/L of Cr and 2.56x10^{-7} mol/L of Ni}) caused no detectable effects in the normal behaviour of rat bone marrow cultures obtained in the experimental conditions described, 1.00x10^{-1}\% \text{SS} impaired osteoblastic behaviour of these cultures and 1.00\% \text{SS} resulted in cell death. Previous work has shown that in rabbit bone marrow cell cultures obtained in similar experimental conditions, stainless steel corrosion products (and also solutions of the corresponding major metal ions prepared from inorganic salts and used at the same concentration) affect proliferation and function of the osteoblast-like cells. Also, metal ions found in others commonly used orthopaedic implants such as Co-Cr-Mo and Ti-6Al-4V alloys were found to cause acute and long-term toxic effects in rat and human bone marrow cultures. Results obtained in this kind of studies suggest that metal ions and particles released from metallic implants may play a role in the bone loss reported to occur surrounding orthopaedic implants.

**CONCLUSIONS**

The present study showed that stainless steel corrosion products above certain concentrations disturb the normal behaviour of rat bone marrow cultures obtained in experimental conditions that favour the proliferation and differentiation of osteoblastic cells.
Metal ions may interfere with the proliferation and differentiation of osteoblastic cells or/and with the function of the differentiated cell. Further studies are required in order to clarify the effect of stainless steel corrosion products in the behaviour of rat bone marrow cell cultures.

References

7. A. Bartolozzi, J. Black, Biomaterials 1985, 6, 2.


4.2- Decreased Consumption of Calcium and Phosphorus during
in Vitro Biomineralisation and Biologically-Induced Deposition of
Nickel and Chromium in the Presence of Stainless Steel Corrosion
Products

ABSTRACT

The purpose of this study was to investigate the effects of 316L stainless steel corrosion products on
the in vitro biomineralisation process, since tissue necrosis, bone loss, impaired bone mineralisation and
loosening of orthopaedic implants had been associated with metal overload. Rat bone marrow cells were
cultured in experimental conditions that favour the proliferation and differentiation of osteoblastic cells
and exposed to stainless steel corrosion products obtained by electrochemical means for periods ranging
from 1 to 21 days. Quantification of total and ionised Ca and P as well as total Fe, Cr and Ni ions in the
culture media of control and metal added cultures during the incubation period was performed in order
to study the influence of corrosion products in the Ca and P consumption that occurs during the
mineralisation process. Control cultures and metal effects on cultures were evaluated concerning DNA
content, enzymatic reduction of MTT, alkaline phosphatase activity, histochemical detection of alkaline
phosphatase and calcium and phosphate deposition and were examined by scanning and transmission
electron microscopy.

The presence of stainless steel corrosion products resulted in an impairment of the normal behaviour
of rat bone marrow cultures. Quantification of metal ions in the culture media and transmission electron
microscopy appearance of the cultures suggested that Cr and Ni appear to be retained by rat bone
marrow cultures. Metal added cultures presented decreased DNA content, MTT reduction and alkaline
phosphatase activity and failed to form mineralised areas; cultures showed negative staining on
histochemical reactions for the identification of calcium and phosphates deposition and scanning and
transmission electron microscopy examination did not show, respectively, mineral globular structures
and mineralisation foci, characteristic of cultures grown in control conditions. These results suggest that
metal ions associated to 316L stainless steel are toxic to osteogenic cells at concentrations approximating
those measured in tissues adjacent to orthopaedic implants.
INTRODUCTION

Ferguson et al.\textsuperscript{1,2,3} presented evidence that all implant metals corroded \textit{in vivo} releasing varying amounts of ions into the local tissues\textsuperscript{4} with the formation of corrosion products which might be transported to distant sites systemically and accumulated in organs\textsuperscript{5}. The local and remote consequences of such release include metabolic, immunologic and carcinogenic effects\textsuperscript{6,7,8}.

Verne et al.\textsuperscript{9} were one of the first groups reporting the use of cell cultures in the qualitative assay of metal toxicity. Since this initial study, quantitative methods had markedly improved allowing a more complete characterisation of corrosion products effects on cells\textsuperscript{10}.

Stainless steel corrosion products obtained by electrochemical means have been a matter of intense research at this laboratory, both in \textit{in vivo} and in \textit{in vitro} studies. Injection in mice of stainless steel corrosion products has shown to cause not only ultrastructural changes in male reproductive organs\textsuperscript{11} and in liver parenchyme\textsuperscript{12}, but also accumulation of multinucleated giant cells and depletion of lymphocyte in spleen\textsuperscript{13}. \textit{In vitro} studies suggested that such corrosion products inhibit the functional activity of human immune cells\textsuperscript{14} and skin fibroblasts\textsuperscript{15} and also impairment in the osteoblastic behaviour of rabbit bone marrow cell cultures\textsuperscript{16}.
The purpose of this study was to investigate the effects of 316L stainless steel (a commonly used orthopaedic material) corrosion products on in vitro biomineralisation process, since tissue necrosis, osteoporosis, bone loss, impaired bone mineralisation\textsuperscript{17-18} and loosening of orthopaedic implants had been associated with metal overload\textsuperscript{19}. Bearing this in mind, quantification of total and ionised Ca and P, and also metal content (Fe, Ni and Cr), in the media of rat bone marrow osteoblast-like cell cultures grown in normal conditions and in the presence of 316L stainless steel degradation products ($8.78 \times 10^{-6}$ mol/L Fe + $4.31 \times 10^{-6}$ mol/L Cr + $2.56 \times 10^{-6}$ mol/L Ni) during culture periods ranging from 1 to 21 days, was performed. Also, osteogenic cell proliferation and function were evaluated using biochemical parameters (enzymatic reduction of MTT, determination of alkaline phosphatase (ALP) activity and DNA content), histochemical assays (identification of ALP positive cells and calcium and phosphate deposits), and examination by scanning and transmission electron microscopy.

Quantification of Ca\textsuperscript{20}, Fe\textsuperscript{21,22}, Ni\textsuperscript{23,24} and Cr\textsuperscript{25} was performed by electrochemical means illustrating the potential of this technique for applications in the biomaterials/biomedical field, since electrochemistry combines a non-destructive character with an extreme sensitivity and inherently good precision\textsuperscript{26}. Moreover, this approach has the particular advantage of being, in principle, a species sensitive method\textsuperscript{26}, not just an element-sensitive method as the traditional technique of atomic absorption spectrometry, providing very significant and versatile opportunities for studies of metal speciation in biological fluids.
MATERIALS AND METHODS

Metallic slurries

A metallic AISI 316L stainless steel (SS) slurry was prepared by anodic dissolution in Hank’s Balanced Salt Solution and the resulting concentrations of the major metal ions were determined by atomic absorption spectrometry: $8.78 \times 10^{-3} \text{ mol/L Fe} + 4.31 \times 10^{-3} \text{ mol/L Cr} + 2.56 \times 10^{-3} \text{ mol/L Ni}$. After pH adjustment (to 7.4) and sterilisation in an autoclave further solutions were obtained by successive dilutions.

Cell culture

A primary culture of osteoblast-like cells was established using a Wistar rat bone marrow suspension obtained accordingly with the method described by Maniatopoulos et al\textsuperscript{27}. Briefly, the femora were excised aseptically, cleaned of soft tissues, and passed through 2 washes, 10 min each, of $\alpha$-MEM (\textit{\alpha}-Minimum Essential Medium, SIGMA M 0894) containing antibiotic antifungal solution (SIGMA A 9909, 1700 units of penicillin per mL, 1.7 mg/mL of streptomycin and 4.2 $\mu$g/mL of amphotericin B). Then, the epiphyses were removed, and the marrow flushed out using a 10 mL syringe with a narrow needle containing culture medium. The cell suspension obtained was distributed, after homogenisation, through three 50 mL culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2} and 95% air. Medium was changed 24 hours later to remove non-adherent cells. The culture medium consisted of $\alpha$-MEM
supplemented with 10% foetal bovine serum, antibiotic antimycotic solution (170 units of penicillin per mL, 0.17 mg/mL of streptomycin and 0.42 µg/mL of amphotericin B), 10^{-8} \text{ mol L}^{-1} \text{ dexamethasone}, 2.52 \times 10^{-4} \text{ mol L}^{-1} \text{ ascorbic acid and 1.00} \times 10^{-2} \text{ mol L}^{-1} \beta\text{-glycerophosphate and was changed twice a week.}

Cells of the first passage (obtained by tripsinisation of cells 7 days after the beginning of the incubation) were seeded at a concentration of 10^4 cells/cm²: a) in 35 mm diameter tissue culture dishes for histochemical assays, scanning electron microscopy (SEM) and transmission electron microscopy (TEM); b) in 24 well culture plates for alkaline phosphatase activity assay and DNA determinations; c) and in 96 wells culture plates for cell viability MTT assay. Cells were cultured in the presence of 0.100% of SS, \textit{i.e.} 8.78 \times 10^{-6} \text{ mol/L Fe + 4.31} \times 10^{-6} \text{ mol/L Cr + 2.56} \times 10^{-6} \text{ mol/L Ni, and in normal culture medium. For control quantification experiments, 35 mm tissue culture dishes containing culture medium with and without added SS corrosion products were incubated and treated as they were cell culture plates. Osteoblast-like cells were cultured during 21 days and tested at days 1, 4, 11, 14 and 21 to perform the biochemical and histochemical assays. The culture media were changed every 2 days, collected and analysed for quantification of total and ionised Ca and P, and also Fe, Cr and Ni.}
**Ions quantifications**

**Ionised and total calcium**

The free and total Ca contents in cell culture media were evaluated by potentiometric means accordingly with the procedure described elsewhere\textsuperscript{20}. The potential was monitored using a calcium selective electrode and an Ag/AgCl reference electrode, and the values of potential were recorded when the readings stabilised within $\pm 0.10$ mV for 5 min. Calibration curves were prepared with standards with a composition similar to the culture medium two-fold and ten-fold diluted. These dilutions were made because it is under these conditions that free (a two-fold dilution) and total (a ten-fold dilution) Ca was quantified in osteoblast-like cell culture medium. The ionic strength of the standard Ca solutions as well as of the samples was adjusted and maintained (during the dilution of the samples) at ca. 0.17 mol/L which corresponds to the value of the ionic strength of culture medium in order to keep the equilibrium of the system unchangeable\textsuperscript{20}.

**Ionised and total phosphorus**

The inorganic (Pi) and total phosphorus (P) were determined colorimetrically using the Fiske and Subbarow procedure\textsuperscript{28}. For Pi quantification, the culture medium was treated with trichloroacetic acid to precipitate protein and lipid-bound phosphates. The supernatant fluid was reacted with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulfite, sodium sulfite and 1-amino-2-naphtol-4-sulfonic acid was then added to reduce the phosphomolybdate to form a
phosphomolybdenum blue complex. The intensity of the colour was measured at 660 nm in a Shimadzu UV-VIS spectrometer.

For P quantification, samples of cell culture medium were, first, digested in a microwave oven (CEM Model MDS-2000) with nitric acid (suprapure 65%) in order to destroy organic matter. Then, the solutions obtained were treated as for Pi quantification (obviously the trichloroacetic acid step was not performed). All values were calculated from standard curves read at the same wavelength.

Iron, chromium and nickel

The quantification of these elements was performed by electrochemical means, after digestion of medium samples, using a mercury film microelectrode, an Ag/AgCl reference electrode and a cylindrical glassy carbon as counter electrode\textsuperscript{21-25}. The technique applied, adsorptive stripping voltammetry, was based on measurement of the current associated with the reduction of the metal-ligand complex adsorbed at the surface of the prepared mercury film microelectrode. For Fe, Cr and Ni analysis in osteoblast-like cell culture medium\textsuperscript{21-25} the organic ligand selected and optimised values for pH, preconcentration potential and time were, respectively,: catechol, \textit{ca.} 8.0, -1.80 V and 30 s, for Fe; diethylenetriaminepenta-acetic acid, \textit{ca.} 6.0, -1.00 V and 10 s, for Cr; and dimethylglyoxime, \textit{ca.} 9.2, -0.70 V and 10 s, for Ni.
Biochemical assays

MTT assay

Cells (8 wells for each situation) were incubated with 0.5 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in the last 4 hours of the culture period tested. The media were then decanted, Formosan salts were dissolved with 100 µL of dimethylsulphoxide and the absorbance was determined at 600 nm in an ELISA reader.

Alkaline phosphatase activity (ALP) and DNA content

Both determinations were performed on the same cultures in order to calculate the ratio ALP/DNA which gives information about the mean ALP content per cell. The following procedure was performed: cultures were collected, rinsed three times with PBS and stored at -20°C.

For the determinations, the samples were placed in 1 mL PBS with 0.05% Triton X-100 and sonified. To 100 µL of this solution, another 100 µL PBS/Triton was added followed by 100 µL of substrate (1x10^{-3} mol L^{-1} paranitrophenylphosphate in 1 mol L^{-1} diethanolamine + 1x10^{-3} mol L^{-1} MgCl_{2}, pH = 9.8). The mixture was incubated at 37°C until the colour was comparable with a standardised series (a 20x10^{-3} mol L^{-1} paranitrophenol solution) in about 15-30 min. The reaction was stopped by the addition of 1 mL 0.1 mol L^{-1} NaOH. All samples including the standardised series were measured in triplicate on a Gilford N-300 photospectrometer at 410 nm.
To the remaining solution we added trypsin in tyrode buffer so that the final trypsin concentration was 1 mg/mL and the mixture was sonified again. To 0.5 mL of the solution, we added 1 mL heparine solution (tromboliqine [Organon 5000 IU, 1:600, PBS]) and 0.5 mL Rnase [Ribonuclease A, Sigma, USA] solution (0.05 mg/mL PBS). The mixture was allowed to react at room temperature for about 0.5-1 hour. Then, 0.5 mL of ethidium bromide solution (0.025 mg/mL PBS) was added. All samples including a DNA-standard series were measured on a Perkin-Elmer LS-3B fluorescence spectrometer at 590 nm\(^3\).

**Histological methods**

a) **Light microscopy**

The presence of ALP positive cells and phosphate or Ca deposits were visualised by histochemical staining. The cultures were fixed with 1.5\% glutaraldehyde in 0.14 mol L\(^{-1}\) sodium cacodylate buffer and rinsed with distilled water.

**ALP staining**

Fixed cultures were incubated during 1 hour in the dark with a mixture, prepared in Tris buffer pH=10, containing 2 mg/mL of Na-\(\alpha\)-naphtyl phosphate (reacting substrate) and 2 mg/mL of fast blue RR salt. The incubation was stopped by rinsing the samples with water and then, the cells were observed in a Olympus BH-2 optical microscope. ALP positive cells stained brown.
Phosphate staining

Phosphate deposits were assessed by the von Kossa technique\textsuperscript{32} \textit{i.e.}, the fixed cultures were covered with a 1.0% silver nitrate solution and kept for 1 hour under UV light. After rinsing, a 5.0% sodium thiosulphate solution was added for 2 min and cultures were washed again. Phosphate deposits stained black.

Calcium staining

The fixed cultures were covered with a 1.0% S alizarin sodium sulfonate solution (0.028% in NH\textsubscript{4}OH), pH=6.4, for 2 min. and then rinsed with water and acid ethanol (ethanol, 0.01% HCl)\textsuperscript{32}. Ca deposits stained red.

b) Scanning electron microscopy

Cultures were fixed as for light microscopy and dehydrated in a graded ethanol series. Critical point drying of the samples was followed by gold sputtering. The preparations were examined in a Philips SEM 525M at 15 kV.

c) Transmission electron microscopy

Cultures were fixed with as for light microscopy and, post-fixed in an aqueous solution of 1.5% potassium ferrocyanide and 1.0% osmium tetroxide for 16 hours at 4\textdegree{}C. Specimens were dehydrated through a graded series of ethanol and embedded in Epon ultra-thin sections.
All data presented correspond to the mean of, at least, three measurements of the same sample and the differences observed between groups were considered significant for $p$ values lower than 0.05 when analysed statistically using the double-sided $t$-test.

**RESULTS**

Rat bone marrow was cultured in experimental conditions reported to favour the proliferation and differentiation of osteoblastic cells in several bone cell culture systems, namely, in the presence of ascorbic acid, $\beta$-glycerophosphate and dexamethasone$^{27,33-37}$. The first subculture was used to study the effects of the SS corrosion products (8.78x10$^{-6}$ mol/L Fe + 4.31x10$^{-6}$ mol/L Cr + 2.56x10$^{-6}$ mol/L Ni) in the *in vitro* biomineralisation process of rat bone marrow osteoblastic cells cultured for periods ranging from 1 to 21 days.

*DNA assessment and MTT reduction*

DNA assessment was used to estimate cell proliferation in cultures grown in control culture medium and in the presence of 0.100% of the SS corrosion products (Fig. 1). Cell proliferation did not increase significantly in the first 10 days of culture and was similar in cultures grown in the two experiments situations, suggesting that during this period the cells were adapting to the culture conditions and that some cells probably died (lag phase). After that, cell growth was exponential and a stationary phase was
observed by the third week of culture. DNA content was significantly lower in metal added cultures as compared to that found in control cultures.

![Graph showing DNA content over time](image)

**Figure 1** - DNA content of rat bone marrow cultures grown in control conditions and in the presence of SS corrosion products for 1 to 21 days. (●) control; (□) 0.100% SS.

* Significant differences (p<0.05) from control.

Despite the controversy and the lack of knowledge related to the enzyme systems which reduce MTT and the localisation of this reaction inside the cells\(^3\), the MTT assay has been widely used to measure cell viability/proliferation and to estimate drugs and biomaterials toxicity\(^39-41\). Results presented in Fig. 2 showed that reduction of MTT in control and treated cultures follows a similar pattern than that observed for DNA content (a lag phase of about 10 days, followed by a exponential increase of the values observed
until approximately day 21). The presence of 0.100% SS corrosion products in rat bone marrow cultures caused a significant decrease in the values observed for the MTT reduction.

![MTT assay graph with time (days) on the x-axis and MTT assay (A=560 nm) on the y-axis.](image)

**Figure 2**- MTT reduction in rat bone marrow cultures grown in control conditions and in the presence of SS corrosion products for 1 to 21 days. (●) control; (□) 0.100% SS.

* Significant differences ($p<0.05$) from control.

As shown in Figs 1 and 2, the two assays provided comparable information, as cellular responses measured are related.
Alkaline phosphatase activity

ALP activity has been routinely used in in vitro experiments as a relative marker of osteoblast differentiation and it is generally accepted that as the specific activity of this enzyme in a population of bone cells increases there is a corresponding shift to a more differentiate state\(^{42}\).

ALP levels in cultures grown in control conditions were relatively low in the first 10 days of culture (in this phase, cell proliferation was also low) and then rise sharply until day 21 (Fig. 3). Fig. 4 shows the results concerning activity of ALP expressed in nmol/min/\(\mu g\) DNA which gives information about the mean ALP content per cell. Values increased during the incubation period suggesting a progressive osteoblastic differentiation of bone marrow cells. Activity of ALP is markedly lower in cultures exposed to 0.100\% of the SS slurry (at day 21 levels of this enzyme were, respectively, 151 nmol/min/cm\(^2\) and 6 nmol/min/cm\(^2\) in control and treated cultures, Fig. 3).

Activity of ALP expressed in nmol/min/\(\mu g\) DNA was very low throughout the 21 days culture period varying from 0.30 to 20.8, and from 0.27 to 1.01 for control and corrosion products treated cultures, respectively.
Figure 3- Alkaline phosphatase activity (nmol/min/cm²) in rat bone marrow cultures grown in control conditions and in the presence of SS corrosion products for 1 to 21 days. (●) control; (□) 0.100% SS.
* Significant differences (p<0.05) from control.

Figure 4- Alkaline phosphatase activity expressed in nmol/min/μg DNA (ALP/DNA) in rat bone marrow cultures grown in control conditions and in the presence of SS corrosion products for 1 to 21 days. (●) control; (□) 0.100% SS.
* Significant differences (p<0.05) from control.
Quantification of total and ionised Ca and P, and Fe, Ni and Cr in the culture media

Culture media from cultures grown in control conditions and in the presence of 0.100% SS corrosion products were collected every two days, throughout the incubation period and analysed for the quantification of total and ionised Ca and P and also, Fe, Cr and Ni ions. Quantification of these species in control and treated culture media incubated in the absence of bone marrow cells in the same experimental conditions as cell cultures was also performed. Results are presented in Figs 5 and 6. Levels measured of total and ionised Ca and P and metal ions were not cumulative, as culture medium was totally replaced every two days; values shown reflect changes occurring in intervals of two days throughout the culture period.

Total and ionised calcium and phosphorus

In the absence of bone cells, levels of total (Fig. 5a) and ionised Ca (Fig. 5b) and P (Fig. 5c and Fig. 5d) in control and metal treated culture media were similar throughout the incubation period. Total Ca concentration was approximately 2 mmol/L and most of it ionised (ca. 1.3 to 1.8 mmol/L). Concentration of total P was around 9-12 mmol/L and concentration of Pi was significantly lower (ca. 1.2 to 1.7 mmol/L) (Fig. 5).

Culture medium collected from control cultures showed increasing concentrations of Pi in the first two weeks of culture (maximal values were 9.00±0.014 mmol/L), although concentration of total P remained constant. Levels of total and ionised Ca did not change significantly during this phase of the culture. However, after approximately two weeks of culture, levels of total Ca (Fig. 5a) and P (almost all ionised at this stage, Fig. 5c)
Figure 5- Levels of total and ionised calcium (a, b) and phosphorus (c, d) in the culture media from control and metal added cultures, collected every 2 days throughout the 21 days incubation period. Levels of these species in the normal and metal treated culture media incubated in the absence of cells is also shown. (○) control culture medium; (●) culture medium from control cultures; (■) 0.100% SS culture medium; (□) culture medium from 0.100% SS cultures.
in the culture medium decreased significantly. These variations were probably caused by the pronounced reductions verified in the ionised contents of these species.

In cultures grown in the presence of 0.100% SS levels of Pi (Fig. 5d) in the incubation medium increased throughout the culture period, although values found were lower than those observed in control cultures. However, levels of total Ca and P were similar during the 21 days incubation and close to those found in the absence of cells, although a trend for a decrease was observed between days 12 and 16 (Fig. 5a and Fig. 5c).

**Fe, Cr and Ni**

Fig. 6 shows the results concerning the levels of Fe, Cr and Ni in the culture media of control and metal added cultures collected every two days during the 21 days incubation period. In the absence of bone cells, no significant changes were observed in the concentrations of Fe, Cr and Ni measured in the control and treated culture media throughout the incubation period. The addition of the SS slurry to the control medium resulted in a small increase in the Fe content (about 45%) when compared to the increases in Cr and Ni contents (three fold for Cr and five fold for Ni). Concentrations of Fe, Cr and Ni in the culture medium from control cultures were similar to those observed in the absence of cells (no differences were observed at p<0.05), during the 21 days culture period. However, levels of Cr and Ni (but not Fe) in the incubation medium collected from cultures treated with SS corrosion products were significantly (at p<0.05
Figure 6- Levels of Fe (a), Cr (b) and Ni (c) in the culture media from control and metal added cultures, collected every 2 days throughout the 21 days incubation period. Concentrations of these species in the normal and metal treated culture media incubated in the absence of cells is also shown. (O) control culture medium; (*) culture medium from control cultures; (■) 0.100% SS culture medium; (□) culture medium from 0.100% SS cultures.
and $p<0.01$) decreased. Concentrations of these ions were lower since the beginning of the culture (particularly for Ni, the lowest contents were verified in the first 4 days of incubation), increased at day 6 and then, decreased values were similar throughout the experiment.

**Light microscopy. Histochemical assays**

Rat bone marrow cells grown in control conditions showed the formation of three-dimensional nodules that increased in size with incubation time. Cells associated with the nodules exhibited an intense ALP and histochemical staining of this enzyme increased throughout the culture period. Alizarin red and von Kossa assays, for the identification of, respectively, calcium and phosphate deposition, showed positive reactions from two weeks onward; in 21 days cultures, the three-dimensional nodules stained intensively for calcium and phosphates deposition. These results are in agreement with those previously reported$^{27,43-44}$. In metal added cultures, a positive histochemical reaction for ALP was also observed, but mineral deposits were not present in these experimental conditions, even in 21 days cultures (negative results on alizarin red and von Kossa assays). Results are shown in Figs. 7A and 7B.
Figure 7A- Histochemical assays (X100) for the identification of alkaline phosphatase in rat bone marrow cultures grown in control conditions (a) and in the presence of SS corrosion products (b) for 21 days.
Figure 7B- Histochemical assays (X100) for the identification of calcium deposition in rat bone marrow cultures grown in control conditions (a) and in the presence of SS corrosion products (b) for 21 days.
SEM and TEM examination of cell cultures

SEM appearance of cultures grown in control conditions was similar to that described previously in this cell system. Mineral deposits were absent in 11 days cultures but were already noticeable in 14 days cultures and continued to be formed and to grow throughout the culture period; 21 days cultures presented numerous globular structures (Fig. 8a) shown by X-ray microanalysis to contain Ca and P (results not shown). Cultures grown in the presence of SS corrosion products showed no evidence of the formation of globular mineral deposits, at this situation only a network of collagen fibrils was visible in 21 days cultures (Fig. 8b).

TEM micrographs of 21 days bone marrow control cultures (Figs. 9A and 9B) showed the presence of cells with morphological similarities to osteoblasts by having a well developed cytoplasm that was rich in rough endoplasmic reticulum, Golgi complexes and lipid inclusions and also the presence of precipitates in the extracellular space that represent mineralisation, according to the previously reported and illustrated in the cell culture system. Numerous small electron dense foci composed of needle-shaped crystals (HA-like crystallites) in different stages of development were observed in these cultures. These mineralisation foci were absent in cultures exposed to SS corrosion products. Instead, precipitates with an amorphous appearance were visible both in the extracellular space and within the cells. These precipitates represent most probably metal deposition from SS slurry present in the culture medium. However, the morphology of the cells was similar in control and metal added cultures.
Figure 8- SEM appearance of rat bone marrow cultures grown in control conditions (a) and in the presence of SS corrosion products (b) for 21 days. Bar=0.1 mm.
Figure 9A- TEM appearance of rat bone marrow cultures grown in control conditions for 21 days. (a) cell morphology (X24400); (b) (X8100) and (c) (X33900), mineralisation foci with needle-shaped crystals (HA-like crystallites) in the extracellular space.
Figure 9A (continued)
Figure 9A (continued)
Figure 9B- TEM appearance of rat bone marrow cultures grown in the presence of SS corrosion products for 21 days. (a) cell morphology (X33900); (b) (X13800) and (c) (X37800) precipitates (metal deposition) in the extracellular space and within the cells.
Figure 9B (continued)
Figure 9B (continued)
DISCUSSION

Preliminary work in this laboratory has examined the effect of 0.010%, 0.100% and 1.00% of the SS slurry prepared (8.78x10^-3 mol/L Fe + 4.31x10^-3 mol/L Cr + 2.56x10^-3 mol/L Ni) in the behaviour of osteogenic cells derived from rat bone marrow (ionised and total Ca and P, histochemical assays and SEM examination of the cultures)\(^{43}\). Results showed that 0.010% of SS had none or little effect in the osteoblastic behaviour of these cultures and 1.00% of this solution caused cell death. SS slurry at a concentration of 0.100% appears to exert some deleterious effects in this culture system; cell morphology appeared normal in the presence of the metal ions but histochemical assays and SEM examination of the cultures suggested an impairment in the mineralisation process\(^{45}\). Bone tissue \textit{in vivo} is probably not exposed to metal ions at concentrations that cause cell death. However, metal ions at sublethal concentrations can still affect the proliferation and function of osteoblastic cells and interfere with the normal mineral deposition, contributing to implant failure.

The aim of the present study was to assess the effects of 0.100% SS slurry, corresponding to 8.78x10^-6 mol/L Fe + 4.31x10^-6 mol/L Cr + 2.56x10^-6 mol/L Ni in the behaviour of osteoblast-like rat bone marrow cell cultures, at culture periods, ranging from 1 to 21 days. The concentration of the major metal ions present in the SS slurry was in the range of iron, chromium and nickel levels found in tissues adjacent to 316L stainless steel plates and screws\(^{46}\).
The results presented showed that cultures grown in control conditions, i.e. in the presence of ascorbic acid, β-glycerophosphate and dexamethasone present osteoblastic features, as demonstrated by the presence of high activity of ALP and formation of mineralised nodules. This behaviour was evident by the results observed in histochemical and biochemical assays and also SEM and TEM appearance of the cultures, as described in the previous section, and are in agreement with the previously reported for this culture system\textsuperscript{27,43-44}.

In this work, quantification of total and ionised Ca and P in the culture media collected from control and metal added cultures was performed. Results showed that, in the absence of bone cells, most of the Ca in the culture media is ionised and concentration of total P is much higher than the concentration of Pi. Ionised Ca and P originate from calcium and phosphates compounds present in α-MEM (2.40 mmol/L and 0.900 mol/L, respectively) and in foetal bovine serum (ca. 3.8 mol/L and ca. 4.0 mol/L, respectively). Addition of β-glycerophosphate (10.0 mmol/L) was responsible for the increased value observed for total P concentration, as compared to that found for Pi. This ester phosphate provides a potential source of phosphate ions required for the mineralisation process in bone nodules forming cultures (deposition of calcium phosphate)\textsuperscript{47-49}. One can notice that Ca and P content in culture medium changed slightly during the time course of the experiment. This fact may be explained bearing in mind that the culture medium has to be prepared several times during the assay and that is practically very difficult to pipette the culture medium uniformly due to the presence of proteins (which bind Ca and P). So, discrepancies may come basically
from two steps: a) during medium introduction in the culture dishes, and b) during collection of aliquots for quantification. The possibility of the incorrect application of the selected methods may be discarded since good precision and accuracy were obtained in preliminary comparative studies and also, other authors reported similar variations in Ca and P content using other techniques. Furthermore, it can be observed that total Ca and P concentrations are, in majority, lower than the expected theoretical values based upon the proportion of each in the culture medium (α-MEM + 10% foetal bovine serum + 10.0 mmol/L β-glycerophosphate) i.e., ca. 2.8 mmol/L for Ca and ca. 11.3 mmol/L for P indicating that some continuous precipitation occurs in all culture plates during the 21 days of incubation. However, this non-biological mineral deposition seems to be insignificant when compared with the high consumption of Ca and P observed during the biomineralisation that occurred in control cultures from approximately two weeks onward (Fig. 5).

Culture medium collected from control cultures showed increasing values of Pi in the first two weeks of culture (although, levels of total P remained constant), suggesting that most of the β-glycerophosphate added is converted to inorganic phosphate, most probably by ALP present in these cultures. Since the measured levels of Pi were not cumulative, results showed that cell cultures have a high ability in hydrolysing this compound (levels at day 6 and 8 were, respectively around 7 and 9 mmol/L). Levels of ALP in cell cultures were relatively low during this stage of the culture (as compared with the maximal values observed for this enzyme, Fig. 3) and the results show the efficacy of this enzyme in hydrolysing this substrate, as reported in numerous
studies\textsuperscript{47-49}. After, approximately two weeks, levels of total Ca and P (almost all ionised at this stage of the culture) in the culture media decrease significantly. This consumption of ionised Ca and P (inorganic phosphate) is related to the mineralisation process, known to occur in this cell system from 2 weeks onward, as demonstrated by the histochemical assays (positive results in alizarin red and von Kossa assays after 14 days of culture) and SEM and TEM appearance of the cultures. These results seem to agree with that reported in the literature concerning studies performed in similar culture systems, \textit{i.e.}, cultures forming mineralised bone nodules in these experimental conditions\textsuperscript{27,47-49}. These studies have shown that alkaline phosphatase and organic phosphate play a crucial role in the initiation of the mineralisation process. Studies\textsuperscript{47} performed in the presence and in the absence of the ALP inhibitor levamisole showed that $\beta$-glycerophosphate is utilised as a substrate for this enzyme and that it is rapidly converted to inorganic phosphate, required for the formation of calcium phosphate. In addition, it was also shown that incorporation of Ca in the cell culture occurs during the mineralisation process\textsuperscript{48-49}. The results reported in the work suggest that evaluation of the concentration of total and ionised Ca and P in the incubation medium throughout the culture period may be regarded as a measure of the mineralisation process occurring in these cell cultures, as calcium phosphate deposition in the extracellular space requires consumption of calcium and phosphate ions from the culture medium.

The presence of 0.10\% SS slurry resulted in the impairment of the normal behaviour of rat bone marrow cultures obtained in the experimental conditions described, as demonstrated by the results reported in the previous section. DNA content and MTT reduction were significantly decreased in metal added cell cultures and activity of
alkaline phosphatase, expressed in nmol/min/μg DNA, was very low, throughout the
culture period. These results suggested an impairment in the differentiation of marrow
cells into cells with osteoblastic phenotype, as ALP has been regarded as a marker of
osteoblast differentiation in *in vitro* culture systems.\(^{42}\) Mineralisation was not observed
in cultures exposed to SS corrosion products. These cultures showed negative staining
on histochemical reactions for the identification of calcium and phosphates deposition
and SEM and TEM examination did not show, respectively, mineral globular structures
and mineralisation foci, characteristic of cultures grown in control conditions. In
addition, concentration of total Ca and P did not change significantly throughout the
culture period, as compared with the significant decrease in the concentration of these
species observed in control cultures after 2 weeks of incubation. Hydrolysis of part of
the β-glycerophosphate added occurred in metal treated cultures and levels of inorganic
phosphate in the culture medium increased throughout the culture period. Hydrolysis of
this compound was most probably performed by ALP, as this enzyme is detected in
these cultures, although at low levels, and it is known to have high efficacy in
hydrolysing this ester phosphate. Other phosphatases present in bone cells, *i.e.*, inorganic pyrophosphatase can also contribute to the hydrolysis of β-glycerophosphate.

Levels of Cr and Ni in the incubation medium of cultures exposed to SS corrosion
products were significantly decreased (*ca.* 56% to 72% for Cr and *ca.* 38% to 75% for
Ni), as compared to those observed in the absence of cells, suggesting the existence of
an biologically-induced precipitation of these species. Decreased concentrations were
observed since the beginning of the culture and values were similar throughout the
incubation period (except for metal contents of day 6 of incubation which were markedly higher than other values), showing an almost regular rate of deposition of these metals in the cell cultures (culture medium was replaced every two days). Biologically-induced deposition of Cr and Ni, but not Fe, may probably be associated with the marked increase in the levels of Cr and Ni ions promoted by the addition of SS corrosion products to the culture control medium leading to the formation of more, proportionally, Cr and Ni organometallic complexes as compared to those formed with Fe and those found in the culture medium of control cultures. Furthermore, as shown by other authors, uptake of these species from its complexes by cells via a transport system may occur with consequent interferences in the cellular metabolism. Also, it may be hypothesised that metabolites produced by bone cells bind Cr and Ni preferentially promoting a sort of selective precipitation, in this case, an extracellular precipitation. These two pathways, separately or together, can both contribute to the decreased Cr and Ni content observed in cell cultures. Chemical precipitation appears not to contribute significantly to the formation of this precipitate, since in the absence of cells only a slight variation on the levels of these metal ions was observed. TEM appearance of metal added cultures supports this view, as precipitates of an irregular electron-dense material were visible in the extracellular space and within the cells. Previous work has shown that human skin fibroblast cultures exposed to the same SS slurry at similar concentrations accumulate Cr and, specially, Ni, as demonstrated by the quantification of these ions in these cultures.

The results presented in this work confirmed preliminary observations showing that SS corrosion products above certain concentrations have deleterious effects in
osteogenic rat bone marrow cultures. Studies performed in similar experimental conditions showed that SS corrosion products also affect proliferation and function of rabbit bone marrow cultures in a dose- and time dependent manner. Also, ions found in other commonly metal alloys (Ti-6Al-4V and Co-Cr-Mo) cause acute and long term toxic effects on osteoblastic cells obtained from rat bone marrow cultured in experimental conditions similar to those used in this work.

Corrosion and fretting have been reported to occur at the interface biological tissues/biomaterial and the high surface area associated with metallic wear debris increases the potential for ion release into the physiological environment. Studies performed in osteogenic cells show that ions found in metallic alloys exert toxic effects upon bone-forming cells suggesting that metals may contribute to peri-implant osteolysis by impairing normal osteogenesis.

In conclusion, the results reported show that SS corrosion products at concentrations approximating those measured in adjacent tissues of orthopaedic implants affect proliferation and function of osteogenic rat bone marrow cultures and also that Ni and Cr appear to be retained by these cultures.

REFERENCES


II- Chapter 5

5- Effects of 316L Stainless Steel Corrosion Products and Corresponding Separate Major Metal Ions in Osteoblast-Like Cells in Different Stages of Differentiation
5.1- *In Vitro* Osteoblastic Differentiation of Human Bone Marrow Cells in the Presence of Metal Ions

**ABSTRACT**

In this study, human bone marrow was cultured in control conditions, that favour the proliferation and differentiation of osteoblastic cells, for periods up to 21 days, and the effect of AISI 316L corrosion products and the corresponding major separate metal ions (Fe, Cr and Ni) was studied in three different phases of the culture period in order to investigate the effects of metal ions in cell populations representative of osteoblastic cells in different stages of differentiation. Toxicity consequences of the presence of metal ions in bone marrow cultures were evaluated by biochemical parameters (enzymatic reduction of MTT, alkaline phosphatase activity and total protein content), histochemical assays (identification of ALP positive cells and calcium and phosphates deposits) and observation of the cultures by light and scanning electron microscopy. Culture media were analysed for total and ionised Ca and P and also metal ions (Fe, Cr and Ni).

The presence of AISI 316L corrosion products and Ni salt in bone marrow cultures during the first and also the second week of culture significantly disturbs the normal behaviour of these cultures interfering in the lag phase and exponential phase of cell growth and ALP expression. However, the presence of these species during the third week of culture, when expression of osteoblastic functions occurs (mineralisation process), did not result in any detectable effect. Fe salt also disturbs the behaviour of bone marrow cell cultures when present during the lag phase and proliferation phase and a somewhat compromised response between the normal pattern (control cultures) and intense inhibition (AISI 316L corrosion products and Ni salt added cultures) is observed. Fe did not affect the progression of the mineralisation phase. Osteogenic cultures exposed to Cr salt (Cr$^{3+}$) presented a similar pattern as the control ones indicating that this element did not interfere, in the concentration studied, in the osteoblastic differentiation of bone marrow cells.

Quantification of metal ions in the culture media showed that Cr (originated from AISI 316L corrosion products but not Cr$^{3+}$ salt) and Ni (originated from AISI 316L corrosion products and Ni salt) appear to be retained by the bone marrow cultures.
INTRODUCTION

The principal applications of AISI 316L stainless steel in the biomedical field are bone and joint replacement, spinal instrumentation and fracture fixation\(^1\). In the vast majority of implant applications, AISI 316L performs its intended function, due to its superior corrosion resistance in addition to good mechanical properties\(^1\), without important adverse physiological effects and have consequently achieved a large degree of success\(^1\). However, clinical experience\(^2\) has revealed that in the presence of a severe and prolonged corrosion process provoked by the aggressive human body environment, inhibition of normal metabolism\(^3,4\), toxic reactions\(^5,6\) and alteration of tissue synthesis and structure can occur. Furthermore, all the major alloying elements of AISI 316L (Fe, Cr and Ni) are essential in the human diet but when their levels increase beyond physiological limits, the precise biochemical balance is disturbed with consequent toxicity\(^7\).

Bone repair (regenerative response to fracture) and remodelling (continuous cycle of destruction and renewal of bone) are carried out by osteoclasts and osteoblasts. Resorption of old bone is accomplished by osteoclasts and subsequent formation of new bone is done by osteoblasts\(^8\). These two processes are highly coupled to each other and are responsible for the renewal of the skeleton while maintaining its anatomic and structural integrity\(^9\). Under normal physiologic circumstances, bone repair and remodelling proceeds in highly regulated cycles\(^9\). However, the presence of corrosion products may affect the systemic and local factors that regulate osteoblastic

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development and their function promoting osteolysis surrounding metal implant. Also, one needs to bear in mind that osteoblasts occur in a continuum of many different stages of differentiation, and that functional behaviour and also response to changes in the local conditions (i.e., presence of drugs) depend on the stage of differentiation in the osteoblastic lineage\textsuperscript{10,11}.

In this study, human osteoblastic cells derived from bone marrow were cultured in control conditions for periods up to 21 days and the effect of metal ions was studied in three different phases of the culture period. Metal treated bone marrow cells were cultured during 7 days (in the presence of metal ions), 14 days (exposed to metal ions during the second week of culture) and 21 days (exposed to metal ions during the third week of culture). All cells, except those used as control, were exposed during seven days to AISI 316L corrosion products and to the corresponding separate major metal ions in the same concentration, in different phases of the culture period, in order to investigate the effects of metal ions in cell populations representative of osteoblastic cells in different stages of differentiation. Toxicity consequences of the presence of metal ions in human bone marrow cultures were evaluated by biochemical parameters (enzymatic reduction of MTT, alkaline phosphatase (ALP) activity and total protein content), histochemical assays (identification of ALP positive cells and calcium and phosphates deposits) and observation of the cultures by light microscopy and scanning electron microscopy (SEM) coupled with X-ray microanalysis. The culture media were analysed for total and ionised Ca and P and also metal ions (Fe, Cr and Ni). Ca and metal contents were determined using a selective electrode\textsuperscript{12} and a mercury microelectrode\textsuperscript{13-16}, respectively, showing that electrochemical methods provide a very
inexpensive and reliable alternative to the traditional method of atomic absorption spectrometry (AAS), combining a non-destructive character with high sensitivity and accuracy.

**MATERIALS AND METHODS**

**Metallic slurries**

Type AISI 316L stainless steel (Fe 63.9%, Cr 18.0%, Ni 12.5%, Mo 2.8%, Si 1.2%, Mn 1.6%, P 0.045% and C 0.025%, weight for weight) was anodically dissolved in Hank’s Balanced Salt Solution (HBSS), which simulates the composition of physiological fluids. The resulting concentrations of the major metal ions in the stock AISI 316L slurry were determined by atomic absorption spectrometry (AAS): 1.02×10⁻² mol/L Fe, 2.67×10⁻³ mol/L Cr and 1.95×10⁻³ mol/L Ni.

Salt solutions containing 8.95×10⁻³ mol/L Fe(FeCl₃.6H₂O), 2.35×10⁻³ mol/L Cr (CrCl₃.6H₂O) or 1.72×10⁻³ mol/L Ni (Ni(NO₃) were prepared separately in HBSS.

**Cell culture**

Osteogenic cells were obtained from bone marrow from a 35 years old man who was undergoing orthopaedic corrective surgery. Bone marrow was cultured in α-MEM supplemented with 10% foetal bovine serum, 2.5 μg/mL fungizone and 50 μg/mL
gentamicin, $10^{-8}$ mol L\(^{-1}\) dexamethasone, $2.52\times10^{-4}$ mol L\(^{-1}\) ascorbic acid and 
$1.00\times10^{-2}$ mol L\(^{-1}\) $\beta$-glycerophosphate\(^{17}\) (control medium). Human bone marrow cells 
were cultured at 37\(^\circ\)C, in a humidified atmosphere containing 5% CO\(_2\) and 95% air. 
Medium containing non-adherent cells was removed 24 hours later, and passages were 
done after trypsinisation of adherent cells in exponential growth (7 days after the 
beginning of the incubation). Cells of the second passage were seeded at a concentration 
of $10^4$ cells/cm\(^2\) in: a) 35 mm diameter tissue culture dishes for histochemical assays and 
SEM observation, b) 96 well culture plates for cell viability MTT assay, ALP activity 
assay and total protein content, and c) 60 mm diameter tissue culture dishes for Ca, P 
and metal quantification in the culture medium.

Bone marrow cells were cultured for periods up to 21 days in control conditions and 
in the presence of AISI 316L corrosion products (SS) or the corresponding separate 
metal ions. Metal treated cultures were exposed to (SS) -$8.95\times10^{-6}$ mol/L Fe + 
$2.35\times10^{-6}$ mol/L Cr + $1.72\times10^{-6}$ mol/L Ni- (obtained by dilution of the stock 316L 
slurry) and Fe, Cr and Ni salt solutions which were added separately to other culture 
dishes at equivalent concentrations, i.e.: $8.95\times10^{-6}$ mol/L, $2.35\times10^{-6}$ mol/L and 
$1.72\times10^{-6}$ mol/L Ni, respectively. The effects of metal ions were studied in different 
stages of the culture period. Cells were cultured for 7, 14 and 21 days in four 
experimental situations. In the first assay, cells were cultured for 7 days and metallic 
elements were added 24 h after trypsinisation (to allow for cell adhesion); cells were 
tested 1 and 7 days later. In the second assay, cells were cultured for 14 days and grew in 
control medium during the first 6 days and were exposed to metallic species between 
days 7-14; cells were tested at days 7 (before metallic exposition) and 14 (after metallic
exposition). In the third assay, cells were cultured for 21 days and grew in control medium during the first 13 days and were exposed to the corrosion products and metal salts between days 14-21; cells were tested at days 7, 14 (before introduction of slurries) and 21 (after metallic exposition). The common feature of the three assays is that cells were exposed to metallic slurries (metal treated cultures) or control medium (cultures used as control) during seven days without renewal of medium. Finally, in the last assay, cells were fed with control medium during 21 days and tested at days 7, 14 and 21. In all experiments, medium was changed twice a week except during the incubation periods in which the effect of the metallic species were studied. Also, every time the medium was changed its content in total and ionised Ca and P, Fe, Cr and Ni was determined. For control quantification experiments, 60 mm tissue culture dishes containing culture medium with and without corrosion products and metal ions added were incubated, treated and analysed as they were cell culture plates (in the different experimental situations studied).

**Biochemical assays**

**MTT assay**

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple Formosan product) was used to estimate cell viability and proliferation. Cells (8 wells for each situation) were incubated with 0.5 mg/mL of MTT in the last 4 hours of the culture period tested. The media were then decanted, Formosan salts were dissolved with 100 μL of dimethylsulphoxide and the absorbance was determined at 600 nm in a ELISA reader.
Alkaline phosphatase activity

ALP was assayed based on the hydrolysis of p-nitrophenylphosphate (used as substrate) into p-nitrophenol at 37°C and pH=10.3. After removal of culture medium, the tissue was washed twice with PBS (phosphate buffered solution and 100 µL of deionised and distilled water with 0.1% Triton X-100 was added to each well. Then, 80 µL of substrate (a 25 mmol L⁻¹ solution) was added to 100 µL of each sample and the mixture was incubated at 37°C during 30 min. The reaction was stopped by the addition of 20 µL of NaOH 5 mol L⁻¹. Optical density was determined at 405 nm on a ELISA reader and compared with the values of a series of p-nitrophenol standards.

Histological methods

a) Light microscopy

The presence of ALP positive cells and phosphate or Ca deposits were visualised by histochemical staining. The cultures were fixed with 1.5% glutaraldehyde in 0.14 mol L⁻¹ sodium cacodylate buffer and rinsed with distilled water.

ALP staining

Fixed cultures were incubated during 1 hour in the dark with a mixture, prepared in Tris buffer pH=10, containing 2 mg/mL of Na-α-naphtyl phosphate (reacting substrate) and 2 mg/mL of fast blue RR salt; the incubation was stopped by rinsing the samples with water. ALP positive cells stained brown.
Phosphate staining

Phosphate deposits were assessed by the von Kossa technique\textsuperscript{18} \textit{i.e.}, the fixed cultures were covered with a 1.0\% silver nitrate solution and kept for 1 hour under UV light. After rinsing, a 5.0\% sodium thiosulphate solution was added for 2 min. and cultures were washed again. Phosphate deposits stained black.

Calcium staining

The fixed cultures were covered with a 1.0\% S alizarin sodium sulphonate solution (0.028\% in NH\textsubscript{4}OH), pH=6.4, for 2 min. and then rinsed with water and acid ethanol (ethanol, 0.01\% HCl)\textsuperscript{18}. Calcium deposits stained red.

b) Scanning electron microscopy and X-ray microanalysis

Cultures were fixed as for light microscopy and dehydrated in a graded ethanol series. Critical point drying of the samples was followed by gold sputtering. The preparations were examined in a JEOL JSM-35C microscope coupled with TRATOR TN-2000 system at 15 keV.

Ions quantification

Ionised and total calcium

The free and total Ca contents in cell culture media were evaluated by potentiometric means accordingly with the procedure described elsewhere\textsuperscript{12}. The potential was monitored using a calcium selective electrode and an Ag/AgCl reference
electrode, and the values of potential were recorded when the readings stabilised within ± 0.10 mV for 5 min. Calibration curves were prepared with standards with a composition similar to the culture medium two-fold and ten-fold diluted. These dilutions were made because it is under these conditions that free (a two-fold dilution) and total (a ten-fold dilution) Ca was quantified in osteoblast-like cell culture medium. The ionic strength of the standard Ca solutions as well as of the samples was adjusted and maintained (during the dilution of the samples) at ca. 0.17 mol/L which corresponds to the value of the ionic strength of culture medium in order to keep the equilibrium of the system unchangeable\(^{12}\).

**Ionised and total phosphorus**

The inorganic (Pi) and total P were determined colorimetrically using the Fiske and Subbarow procedure\(^{19}\). For Pi quantification, the culture medium was treated with trichloroacetic acid to precipitate protein and lipid-bound phosphates. The supernatant fluid was reacted with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulfite, sodium sulfite and 1-amino-2-naphtol-4-sulfonic acid was then added to reduce the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the colour was measured at 660 nm in a Shimadzu UV-VIS spectrometer.

For total P quantification, samples of cell culture medium were, first, digested in a microwave oven (CEM Model MDS-2000) with nitric acid (suprapure 65%) in order to destroy organic matter. Then, the solutions obtained were treated as for Pi quantification.
(obviously the trichloroacetic acid step was not performed). All values were calculated from standard curves read at the same wavelength.

Iron, chromium and nickel

The quantification of these elements was performed by electrochemical means, after digestion of medium samples, using a mercury film microelectrode, an Ag/AgCl reference electrode and a cylindrical glassy carbon as counter electrode\textsuperscript{13-16}. The technique applied, adsorptive stripping voltammetry, was based on measurement of the current associated with the reduction of the metal-ligand complex adsorbed at the surface of the prepared mercury film microelectrode. For Fe, Cr and Ni analysis in osteoblast-like cell culture medium\textsuperscript{13-16} the organic ligand selected and optimised values for pH, pre-concentration potential and time were, respectively,: catechol, \textit{ca}. 8.0, -1.80 V and 30 s, for Fe; diethylenetriaminepentaacetic acid, \textit{ca}. 6.0, -1.00 V and 10 s, for Cr; and dimethylglyoxime, \textit{ca}. 9.2, -0.70 V and 10 s, for Ni.

For biochemical data each point represents the mean ± standard deviation of, at least, six replicates and for Ca and P, and also metal ions quantification, data presented correspond to the mean ± standard deviation of, at least, three independent analyses being each individual value obtained by performing three replicates. The differences observed between groups were considered significant for \textit{p} values lower than 0.05 when analysed statistically using the double-sided \textit{t}-test.
RESULTS

Human bone marrow stromal cells were grown in conditions known to favour the formation of osteoblast-rich cell cultures and in the presence of SS (8.95x10^{-6} \text{ mol/L Fe} + 2.35x10^{-6} \text{ mol/L Cr} + 1.72x10^{-6} \text{ mol/L Ni}), Fe salt (8.95x10^{-6} \text{ mol/L}), Cr salt (2.35x10^{-6} \text{ mol/L}) and Ni salt (1.72x10^{-6} \text{ mol/L}). The aim of this work was to investigate the influence of metal elements, commonly present in orthopaedic implants, in the osteoblastic differentiation of human bone marrow cells. For this propose, osteoblast-like cells were cultured for periods up to 21 days in control conditions and exposed to metal ions in different phases of the culture period (i.e., during the first, second, and third week of incubation). In another experiment, bone cells were cultured in control conditions for 21 days. Control and metal treated cultures were evaluated by biochemical and histochemical assays and observed by SEM. Culture media were analysed for total and ionised Ca and P, and also Fe, Cr and Ni ions.

MTT reduction

The MTT test, where tetrazolium salts are transformed by active enzymes into intracellular Formosan deposits, measures cell activation; only viable cells undamaged by biomaterials or any other irritant can express this activity.

Proliferation of cells grown in control conditions for 21 days (Fig. 1a) increased smoothly in the first week, suggesting that during this period the cells were adapting to
the culture conditions and that some of them probably died (lag phase). After that, cell growth was exponential and a tendency for a stationary phase was observed by the third week of culture.

Fig. 1b shows results concerning the reduction of MTT by human bone marrow cells grown for 7 days in control conditions and exposed to metal ions. A significant decrease ($p<0.05$) in cell proliferation was observed in metal treated cultures, specially evident in the presence of SS and Ni ions, suggesting that the presence of these species disturbs the initial stages of the culture, namely the adaptation of bone cells to culture conditions. In cultures grown for 14 days and exposed to metal ions during the second week of culture (Fig. 1c), SS and Ni ions cause a deleterious effect in cell viability/proliferation ($p<0.05$), as measured by the MTT assay, relatively to cells grown in control conditions during the 14 days of incubation. These metal species also promoted a decrease in MTT reduction ($p<0.05$) in cultures grown for 21 days and exposed to metal ions during the third week of incubation (Fig. 1d). Fe and Cr ions only interfere with the normal growth of bone cells in culture when added in the first week of incubation.
Figure 1- MTT reduction in human bone marrow cultures grown in control conditions and in the presence of metallic slurries in different phases of the culture period. (a) Cells grown for 21 days in control conditions; (b) Cells grown for 7 days in the presence of metallic slurries; (c) Cells grown for 14 days and exposed to metallic slurries in the second week of incubation; (d) Cells grown for 21 days and exposed to metallic slurries in the third week of incubation.

Control (■); SS (×); Fe (+); Cr (▲); Ni (●).

* Significant differences (*p*<0.05) from control cultures.
Total protein content

In cultures grown in control conditions for 21 days, total protein increased with the incubation time in a way similar to cell proliferation, remaining approximately constant during the third week of culture (Fig. 2a). Total protein content in cultures grown for 7, 14 and 21 days in control conditions and exposed to SS and the corresponding separate metal ions, in the experimental conditions studied, are presented in Fig. 2b-d. Results show that alterations in total protein content can be observed in the presence of the metallic species, specially in cultures exposed to SS and Ni salt (similar to that observed in the MTT assay with the exception of the slight protein increase, as compared to the control, promoted by Ni salt in the third week of culture; \( p<0.05 \)). However, the effect of the presence of metal ions in the total protein content of bone cell cultures was quantitatively less pronounced than that observed in the MTT reduction in metal treated cultures (maximal suppression values of ca. 16% and ca. 31% in total protein content and MTT reduction, respectively, were observed in cultures grown during 14 days and exposed to metal ions during the second week). Although these two parameters (MTT reduction and total protein content) are a measure of cell proliferation, the information provided is somewhat different. Protein measured in these cultures includes intracellular and extracellular protein and the later contributes significantly for total protein content in bone cell cultures. This probably explains the less pronounced effect of metal ions in cell cultures total protein content.
Figure 2- Total protein content (µg/cm²) in human bone marrow cultures grown in control conditions and in the presence of metallic slurries in different phases of the culture period.

(a) Cells grown for 21 days in control conditions; (b) Cells grown for 7 days in the presence of metallic slurries; (c) Cells grown for 14 days and exposed to metallic slurries in the second week of incubation; (d) Cells grown for 21 days and exposed to metallic slurries in the third week of incubation.

Control (■); SS (∗); Fe (+); Cr (▲); Ni (●).

* Significant differences (p<0.05) from control cultures.
Alkaline phosphatase activity

The identification and characterisation of osteoblast-like cell cultures is based on the assay of specific metabolites. For this purpose, the activity of ALP, a membrane enzyme routinely used in in vitro experiments as a relative marker of osteoblastic differentiation\textsuperscript{21,26} was determined and results are shown in Fig. 3.

ALP levels of bone marrow cells grown in control conditions for 21 days were relatively low in the first week of culture, then rose sharply in the second week to some peak level of response and drop by the third week of incubation (Fig. 3a).

In cultures grown for 7 days, only the presence of SS and Ni ions resulted in a significant reduction in ALP activity ($p<0.05$, 77\% and 58\%, respectively). In cultures grown for 14 days, the presence of SS in the second week of incubation (phase characterised by an exponential increase in ALP activity) resulted in a significant decrease in ALP levels ($p<0.05$, 75\% reduction); cultures exposed to Fe and Ni ions also presented lower levels of ALP in the second week of cultures, as compared to those found in control cultures ($p<0.05$, a reduction of 31\% and 33\%, respectively) and Cr ions cause no detectable effect in the activity of enzyme. In cultures maintained for 21 days, the addition of metal species during the third week of incubation did not affect significantly the behaviour of bone marrow cultures, although in the presence of SS and Ni ions a small decrease in ALP activity was observed.
Figure 3- ALP activity (nmol/min/cm²) in human bone marrow cultures grown in control conditions and in the presence of metallic slurries in different phases of the culture period.
(a) Cells grown for 21 days in control conditions; (b) Cells grown for 7 days in the presence of metallic slurries; (c) Cells grown for 14 days and exposed to metallic slurries in the second week of incubation; (d) Cells grown for 21 days and exposed to metallic slurries in the third week of incubation.
Control (■); SS (×); Fe (+); Cr (▲); Ni (○).
* Significant differences (p<0.05) from control cultures.
Results concerning activity of ALP expressed in nmol/min/µg protein (results not shown) in control and metal treated cultures which give information about the mean ALP content per total amount of protein (intracellular + extracellular) presented similar profiles as those obtained for ALP expressed in nmol/min/cm².

Results presented in Fig. 3 show that SS and Ni salt cause a deleterious effect in ALP activity of bone marrow cultures; Cr salt had practically no effects on the activity of this enzyme and Fe salt cause a small decrease when present in the second week of culture.

Quantification of total and ionised Ca and P, and Fe, Ni and Cr in the culture media

Media from cultures grown in control conditions and in the presence of metallic slurries were collected throughout the incubation period and analysed for the quantification of total and ionised Ca and P, and also Fe, Cr and Ni. Quantification of these species in control and metal ions exposed media incubated in the absence of bone marrow cells was also performed. Levels measured of total and ionised Ca and P, and metal ions were not cumulative, as culture medium was totally replaced twice or once (during the incubation periods in which the effect of the metallic species were studied) a week; values shown reflect changes occurring in intervals of 3/4 or 7 days throughout the culture period. Results are presented in Figs 4-5 and Table 1, respectively.
Total and ionised Ca and P

In the absence of bone cells, levels of total and ionised Ca and P in control and metal treated culture media were similar throughout the incubation periods. Total Ca concentration ranged from a minimum of $2.39 \pm 0.030$ mmol/L to a maximum of $2.61 \pm 0.13$ mmol/L and most of it was ionised ($1.54 \pm 0.027$ mmol/L to $1.90 \pm 0.054$ mmol/L); concentration of total P ranged from $10.8 \pm 0.099$ mmol/L to $11.1 \pm 0.20$ mmol/L and content of ionised P was significantly lower ($1.28 \pm 0.0040$ mmol/L to $2.03 \pm 0.010$ mmol/L).

Ca and P in culture medium originate from calcium and phosphates compounds present in $\alpha$-MEM (2.40 mmol/L and 0.900 mmol/L, respectively) and in foetal bovine serum (ca. 3.8 mmol/L and ca. 4.0 mmol/L, respectively). Addition of $\beta$-glycerophosphate (10.0 mmol/L) to the incubation medium was responsible for the increased value observed for total P concentration, as compared to that found for Pi. This ester phosphate provides a potential source of P ions required for the mineralisation process in bone nodules forming cultures (deposition of calcium phosphate). One can notice that Ca and P content in culture medium changed slightly during the time course of the experiment. This fact may be explained bearing in mind that the culture medium has to be prepared several times during the assay and that is practically very difficult to pipette the culture medium uniformly due to the presence of proteins (which bind Ca and P). The possibility of the incorrect application of the selected methods may be discarded since good precision and accuracy were obtained in preliminary comparative studies and also, other authors reported similar variations in Ca and P.
content using other techniques. Furthermore, it can be observed that total Ca and P concentrations are, in majority, lower than the expected theoretical values based upon the proportion of each in the culture medium (α-MEM + 10% foetal bovine serum + 10.0 mmol/L β-glycerophosphate) i.e., ca. 2.8 mmol/L for Ca and ca. 11.3 mmol/L for P indicating that some precipitation occurs in the stock culture medium flask and/or in all culture plates during the incubation period.

Culture medium collected from cultures grown in control conditions for 21 days (Fig. 4) showed constant levels of total Ca and P, and also ionised Ca during the first 10 days of incubation, although concentration of Pi increased during the first two weeks of culture (maximal values were 9.30 ± 0.083 mmol/L at day 14) suggesting that most of the β-glycerophosphate added is utilised as a substrate by ALP, present in high levels in these cultures. However, after approximately two weeks of incubation, levels of Ca and P started to decrease continuously until the end of the culture. These variations were probably caused by the pronounced reductions verified in the ionised contents of these species and reflect, most probably, the mineralisation process, i.e., the deposition of calcium phosphate in the cultures. These results seem to agree with that reported in the literature concerning studies performed in similar culture systems, i.e., cultures forming mineralised bone nodules in these experimental conditions\textsuperscript{27-29}. Results presented in Fig. 4 show that non-biological mineral deposition occurred in the absence of bone cells seems to be insignificant when compared with the high consumption of Ca and P observed during the biomineralisation that occurred in control cultures from approximately two weeks onward.
Figure 4- Levels of total and ionised Ca (a, b) and P (c, d) in the culture medium from human bone marrow cultures grown for 21 days in control conditions (■). Levels of these species in the culture medium incubated in the absence of cells are also shown (□, dashed line).
No significant variations in total Ca and P, and also ionised Ca were measured in media from control and metal exposed cultures maintained for 7 days. Nevertheless, the level of Pi increased from $1.28 \pm 0.0040$ mmol/L (at day 0) to $4.39 \pm 0.10$ mmol/L in the control situation and to $2.56 \pm 0.044$ mmol/L, $4.73 \pm 0.18$ mmol/L, $4.57 \pm 0.089$ mmol/L, $3.46 \pm 0.012$ mmol/L in the presence of SS, Fe, Cr and Ni slurries, respectively (results not shown).

Results concerning culture maintained for 14 days and exposed to metal ions in the second week are presented in Fig. 5A. As compared to control cultures, Cr treated cultures presented a similar pattern of variation in the levels of total and ionised Ca and P in the culture media; Fe treated cultures presented lower levels of Pi in the second week of incubation and also, a lower consumption of Ca and P from the culture medium (levels of these species in control and Fe treated cultures were significantly different, $p<0.05$). In contrast, media from SS and Ni treated cultures contained as much total Ca and P, and ionised Ca as media incubated in the absence of cells; Pi levels in the culture media were markedly lower during the second week of incubation ($p<0.05$) as compared to the control values.

In cultures maintained for 21 days (Fig. 5B), the addition of SS and the corresponding metal salts during the third week of culture caused no detectable effect in the behaviour of bone marrow cultures concerning levels of total and ionised Ca and P in the culture media.
Figure 5A- Levels of total and ionised Ca (a, b) and P (c, d) in the culture media from metal treated cultures grown for 14 days and exposed to metallic slurries in the second week of incubation. Levels of these species in the culture media incubated in the absence of cells are also shown.

In the presence of cells:
Control (■); SS (+); Fe (▲); Cr (●); Ni (◆).

In the absence of cells (dashed lines):
Control (□); SS (×); Fe (△); Cr (○); Ni (◇).
Figure 5B- Levels of total and ionised Ca (a, b) and P (c, d) in the culture media from metal treated cultures grown for 21 days and exposed to metallic slurries in the third week of incubation. Levels of these species in the culture media incubated in the absence of cells are also shown.

In the presence of cells:
Control (■); SS (+); Fe (▲); Cr (●); Ni (◇).

In the absence of cells (dashed lines):
Control (□); SS (×); Fe (△); Cr (○); Ni (◇).
Fe, Cr and Ni

In control medium, mean ± standard deviation values of Fe, Cr and Ni were, respectively, (1.64 ± 0.070) \times 10^{-5} \text{ mol/L}, (5.63 ± 0.18) \times 10^{-7} \text{ mol/L} and (4.96 ± 0.22) \times 10^{-7} \text{ mol/L}; no significant changes in the concentration of these metal ions were observed during the control assays performed in the presence and absence of cells. Results are in agreement with previously reported studies in rat bone marrow osteogenic cultures\(^{31}\). Addition of SS to the control culture medium increased the Fe, Cr and Ni concentrations to (2.54 ± 0.040) \times 10^{-5} \text{ mol/L (ca. 55%)}), (2.90 ± 0.070) \times 10^{-6} \text{ mol/L (ca. four fold)} and (2.21 ± 0.060) \times 10^{-6} \text{ mol/L (ca. three fold)}, respectively. Similarly, addition of metal salts to the control culture medium rose the metal contents to (2.54 ± 0.020) \times 10^{-5} \text{ mol/L for Fe, (2.94 ± 0.050) \times 10^{-6} \text{ mol/L for Cr and (2.23 ± 0.070) \times 10^{-6} \text{ mol/L for Ni.}}

Table 1 shows the variations of Fe, Cr and Ni contents measured in SS treated culture media collected from bone marrow cultures maintained for 7, 14 and 21 days in the experimental conditions studied. In the absence of cells, metal contents appeared to be approximately constant in the three situations investigated. In contrast, a different pattern occurred in the presence of osteogenic cells; substantial reductions in the Cr and Ni concentrations were verified.

Results concerning the variations of Fe, Cr and Ni contents in the culture medium from cultures exposed to the metal salts are also presented in Table 1. In the absence of cells, a similar pattern as the one observed in SS treated cultures media was obtained,
Table 1- Percentage of variation of Fe, Cr and Ni contents in the media of cultures treated with AISI 316L corrosion products and the corresponding separate major metal salts, in the absence or in the presence of human bone marrow cells (relative to the concentrations that were measured after the addition of AISI 316L corrosion products or the corresponding metal salts to the culture medium).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Fe (%)</th>
<th>Cr (%)</th>
<th>Ni (%)</th>
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<td>Fe salt treated cultures</td>
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<tr>
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</tbody>
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*Maximal relative standard deviations obtained for Fe, Cr and Ni concentrations were 4.5%, 3.5% and 4.5%, respectively.

*Key:
1- Cells grown for 7 days;
2- Cells grown for 14 days and exposed to metal ions during the second week;
3- Cells grown for 21 days and exposed to metal ions during the third week.
i.e., no significant changes in the amount of metal ions were measured. However in the incubation media from osteoblastic cultures, only the Ni levels were markedly lower.

**Histochemical assays. SEM examination of cell cultures.**

Human bone marrow cells grown in control conditions showed the formation of a nodular structure that increases in size with incubation time. Cells associated with the nodules presented intense histochemical staining for ALP; in 21 days cultures, the three-dimensional nodules stained intensively for calcium and phosphates deposition, as demonstrated by a strong positive reaction in Alizarin red and von Kossa assays, respectively. SEM examination of 21 days cultures (Fig. 6a) shows the presence of numerous globular structures, shown by X-ray microanalysis to contain Ca and P (Fig. 6b) in a ratio of $1.7 \pm 0.06$ (n=10), which is in agreement with the Ca/P ratio of the hydroxyapatite (HA). Bone marrow cells grown for 7 days in the presence of Fe and Cr ions showed a similar behaviour than that observed in control conditions but exposition to SS and Ni ions resulted in evident signs of deterioration of the cultures, demonstrated by a significative decrease in the number of cells, and the remaining cells presented alteration in cell morphology (cells became bigger and more spreaded) and a weak histochemical reaction for ALP. Also, in cultures grown for 14 days, the presence of SS in the second week resulted in a similar deleterious effect in the behaviour of bone marrow cells; no marked differences were observed in the histochemical reactions and SEM appearance of the cultures treated with Fe and Cr salts, as compared with control cultures. In 21 days cultures, the presence of SS and the corresponding separate metal salts during the third week of culture did not result in apparent changes in the
Figure 6- (a) SEM appearance of human bone marrow cultures grown for 21 days in control conditions. Bar=10 μm. (b) Energy dispersive X-ray microanalysis spectrum of the three-dimensional globular deposits showing the Ca and P peaks.
histrochemical behaviour and SEM appearance, as compared to cultures grown in control conditions.

**DISCUSSION**

In this study, the combination of biochemical assays such as enzymatic reduction of MTT, ALP activity and total protein content with chemical analyses of culture media, concerning Ca, P, Fe, Cr and Ni concentrations by spectrophotometry and electrochemistry, and with morphological assays enabled us to characterise the differential cellular response, depending on the stage of differentiation of the target osteoblasts, to 0.100% of a stainless steel slurry (8.95x10⁻³ mol/L Fe + 2.35x10⁻³ mol/L Cr + 1.72x10⁻³ mol/L Ni) and to its individual major compounds ( slurries of Fe, Cr and Ni salts in the concentration present in the AISI 316L slurry). This concentration of corrosion products was selected since previous studies at this laboratory had examined the dose- and time-effects of a)- 0.001%, 0.010% and 0.100% of this AISI 316L slurry and corresponding separate major metal ions on rabbit osteogenic cell proliferation and function⁴², and of b)- 0.010%, 0.100% and 1.00% of another AISI 316L slurry (8.78x10⁻³ mol/L Fe + 4.31x10⁻³ mol/L Cr + 2.56x10⁻³ mol/L Ni) in the behaviour of osteogenic cells derived from rat bone marrow⁴¹,⁴³. And, a common feature of both preliminary investigations, was that 0.001% and 0.010% of the slurries had none or little influence in the osteoblastic behaviour and that 1.00% caused cell death⁴³. AISI 316L slurry at a concentration of 0.100% appeared to exert some deleterious effect in the rat culture system⁴¹,⁴³. Bone tissue in vivo is probably not exposed to metal ions at
concentrations that cause cell death. However, metal ions at sublethal concentrations can still affect the proliferation and function of osteoblastic cells and interfere with the normal mineral deposition, contributing to implant failure.

The selected biochemical parameters showed that the human bone marrow cell cultures grown in control conditions, *i.e.* in the presence of ascorbic acid, β-glycerophosphate and dexamethasone present osteoblastic features, as demonstrated by the presence of high activity of ALP (Fig. 3a) and formation of mineralised nodules. These cultures presented a similar behaviour concerning cell proliferation and osteoblastic differentiation to that reported in other bone nodules forming cultures¹¹,¹³,¹⁷,²⁷,³⁴-³⁶. After a lag phase of approximately one week, bone marrow cells enter a period of active proliferation that is functionally related to the synthesis of a bone-specific extracellular matrix during the second week of incubation and after, on the third week, a tendency for a stationary phase in cell growth is observed (as a consequence of the accumulation and maturation of the extracellular matrix³⁴). ALP expression followed a similar pattern of variation with the incubation time. The significant increase in the levels of this enzyme during the second week of incubation suggested that cells were shifting to a more differentiated state³⁷-³⁹. ALP appears to play a crucial role in the initiation of matrix mineralisation and, after that, expression of this enzyme is down regulated¹¹,¹³,²⁷,³⁵-³⁶. The mineralisation process, an osteoblast specific function, occurred during the third week of culture, as demonstrated by the strong positive reactions for alizarin red and von Kossa assays, the presence of numerous mineral globular structures on SEM observation and also a significative consumption of ionised Ca and P from the culture medium during this phase of the culture.
The presence of SS and Ni salt in bone marrow cultures during the first and also the second week of culture significantly disturbs the normal behaviour of these cultures. These results suggest that these slurries impair the initial stage of the culture, i.e., the lag phase (first week), probably interfering with the adhesion process of bone cells; also, they exert toxic effects during the exponential phase of cell growth and ALP expression (second week), disturbing the proliferation phase that is associated with the formation of extracellular matrix. According to this, cultures treated with these metallic species presented a weak ability to ionise the β-glycerophosphate added (probably because of the lower levels of ALP existing in these cultures) and no consumption of Ca and P from the culture media was observed, as compared to control cultures. Results also show that the presence of Ni salt appeared to result in a less pronounced effect, as compared to that observed in the presence of SS. However, it is interesting to note that, despite their toxicity in these stages of the culture, the presence of SS and Ni salt during the third week of culture, when expression of osteoblastic functions occurs (mineralisation process) did not result in any detectable effect. Fe salt also disturbs the behaviour of bone marrow cell cultures when present during the lag phase and proliferation phase (first and second week) and a somewhat compromised response between the normal pattern (control and Cr treated cultures) and intense inhibition (SS and Ni salt added cultures) is observed. Fe did not affect the progression of the mineralisation phase.

Osteogenic cultures exposed to Cr salt (Cr\(^{3+}\)) presented a similar pattern as the control ones indicating that this element did not interfere, in the concentration studied, in the osteoblastic differentiation of bone marrow cells, observation suggested by the
lack of effect concerning MTT reduction (except a small reduction in the first week), ALP activity and also the pattern of variation of Ca and P levels in the incubation medium. In a recent study\textsuperscript{40}, Cr\textsuperscript{3+} was also recognised as having low cytotoxicity in ROS 17/2.8 rat osteoblast-like cells, as compared to other metal ions that can be released from metallic implants \textit{in vivo}, probably because of its membrane impermeability and biologic inactivity. Previous studies\textsuperscript{41} revealed that Cr\textsuperscript{6+} induced the most cytotoxicity response on bone marrow stromal cells cultured \textit{in vitro} as compared with Fe\textsuperscript{3+} and Ni\textsuperscript{2+}. Apparently contradictory results may be explained by the different oxidation state used since the cytotoxicity of soluble and insoluble hexavalent Cr compounds appears to be 100-1000 times greater than that demonstrated by trivalent Cr compounds\textsuperscript{42}.

Results show that metallic slurries did not interfere in the normal behaviour of bone cells cultures when present during the culture period where mineralisation process occurs (third week). In control conditions, mineralisation appears to begin from two weeks onward. An enhanced expression of ALP is needed just before the onset of matrix mineralisation and may well be the triggering event of the calcification cascade\textsuperscript{37,43}, providing localised enrichment of Pi for HA crystal nucleation and proliferation; following the initiation of mineralisation there is a decrease in ALP activity. Thus, cells are more importantly involved in mineral initiation (providing high levels of ALP) while propagation remains primarily physicochemical\textsuperscript{43}; exposition of the initial crystals formed to the rich Ca and P culture medium activates crystal proliferation in what is essentially a physicochemical process\textsuperscript{43}. 

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The pattern of variation for Ni and Cr content in the incubation medium of cultures exposed to SS was similar to that observed previously\textsuperscript{31}; decreased concentrations of the ions were similar throughout the three incubation periods showing an almost regular rate of deposition of these metal ions in the cell cultures\textsuperscript{31}. Media of cultures treated with Ni salt presented the same trend of variation. As demonstrated by other authors\textsuperscript{42,44-46}, uptake of these species from its complexes by cells via a transport system may occur with consequent interference in the cellular metabolism (intracellular metallic deposition). Also, it may be hypothesised that some metabolites produced by bone cells bind Cr (originated from SS) and Ni (originated from SS or Ni salt) preferentially promoting a sort of selective precipitation, in this case, an extracellular precipitation. These two pathways, separately or together can both contribute to the decreased contents measured in cell cultures. Chemical precipitation appears not to contribute significantly to these reductions, since in the absence of cells only slight variations on the levels of these metal ions were observed, suggesting a biologically induced phenomenon. Contradictory to Ni, the Cr salt used (Cr\textsuperscript{3+}) did not reproduce the biological behaviour of Cr compounds that originate from the corrosion of AISI 316L; maximum Cr\textsuperscript{3+} level of variation in the presence of cells was 16.6\% which is not significantly different from the maximum decrease observed in the absence of cells, 17.1\%. Merritt \textit{et al.}\textsuperscript{44} showed that corrosion products from stainless steel and cobalt-chrome alloy behave similarly to the metal salts and that fretting\textsuperscript{47} of AISI 316L in serum resulted in corrosion products containing Cr\textsuperscript{6+}. Cr\textsuperscript{6+} is able to penetrate the cell membrane, but not trivalent Cr which is excluded (except at concentrations 100 or 1000 times those for the hexavalent form)\textsuperscript{42,48}. Since Cr can exist in a variety of states, the possibility of several Cr valence states also existed; however, these other valence states will not be near as toxic as the
Cr$^{6+}$ \(^{49}\). These hypotheses may help to explain the cytotoxicity of SS, in combination with previous results obtained by Lucas et al.\(^{49}\) that indicate in vitro induction of cellular ultrastructural damage by Ni, and also the low levels of Cr found in culture media exposed to SS and osteogenic cells.

Although the primary constituents of AISI 316L are biologically essential trace elements, implantation of a device containing this alloy will constitute a long-term in vivo source of reactive trace elements, any of which may produce bone sequel when introduced at levels not physiologically regulated. Furthermore, metal species react in biological systems and as different species are formed, significant changes in toxicity may occur.

Results of this study have emphasised that corrosion products have differential effects, depending on the state of differentiation of the target cells and also that, understanding of these processes will be incomplete until the relative proportions and different chemical species (of the same element) formed during implant degradation will be determined/characterised.

**REFERENCES**

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22. Y. Gotoh, K. Hiraiwa, M. Narajama, Bone and Min. 1990, 8, 239.


III- MAIN CONCLUSIONS AND FINAL REMARKS
MAIN CONCLUSIONS AND FINAL REMARKS

The initial promise of this work, which was to contribute for the development and application of electrochemical quantification techniques to the biomedical research field, has been accomplished. Methodology, using microelectrodes in which a mercury film is deposited coupled with stripping voltammetry, has been developed following the trends of the last decade of electroanalytical techniques, *i.e.*, "smaller and faster"\(^1\), to measure easily, rapidly, reproducibly and accurately low concentrations of iron, chromium and nickel in cell culture medium. However, voltammetric responses must always be carefully characterised since adsorptive stripping voltammetry analysis sometimes lack the predictability of techniques such as spectrometry\(^2\), mostly because of the relative selectivity of the complexing agents which bind either to the target ion to be determined or to other ions and some residues of organic matter that may still remain in the samples after the digestion process. In this study, breakthroughs in selectivity and sensitivity were achieved by careful optimisation of all the operational parameters, starting in the digestion procedure and ending with the application of the standard additions method, and the results obtained, for each metal ion, were: i) catechol 5.00x10\(^{-4}\) mol/L, PIPES buffer 1.00x10\(^{-2}\) mol/L (pH=8.0), deposition potential -1.80 V (vs. Ag/AgCl) and detection limit 1.93x10\(^{-8}\) mol/L for a collection time of 30 s, for iron; ii) diethylenetriaminepentaacetic acid 10.0x10\(^{-3}\) mol/L, sodium nitrate 0.700 mol/L, sodium acetate 4.00x10\(^{-2}\) mol/L (pH=6.0), potassium permanganate 1.00x10\(^{-3}\) mol/L, deposition potential -1.00 V (vs. Ag/AgCl) and detection limit 2.80x10\(^{-10}\) mol/L for a collection time of 40 s, for chromium; iii) dimethylglyoxime 5.00x10\(^{-4}\) mol/L,
ammonium chloride buffer 0.100 mol/L (pH=9.2), deposition potential -0.70 V (vs. Ag/AgCl) and detection limit 7.70x10^{-9} mol/L for a collection time of 10 s, for nickel.

Ion-selective electrodes which are included in the strongest group of the chemical sensors, *i.e.* potentiometric sensors\(^3\), are robust devices for total and ionised calcium measurements in cell culture medium with minimum interference from proteins, polyelectrolytes and colloidal materials (destruction of organic matter and pH adjustment to *ca.* 6.5 or higher will obligate to a dilution of at least ten times, before measurements, to prevent that most of the calcium, as well as magnesium and phosphorus, precipitate). After appropriate calibration (*i.e.* based on standards with a composition similar to the culture medium ten-fold or two-fold diluted, a pH of 9.6 or 7.4, for total and ionised calcium quantification, respectively, and also, an ionic strength of 0.17 mol/L), concentrations can be read directly and rapidly. In contrast to other fields of applications, however, in biological systems the danger always exists that the measuring process may disturb the physiological equilibrium.

Although it is worthwhile to emphasise the significance of the extension of these electrochemical methods to the biomedical/biomaterials domain, it is not intended to over-rate them here. By no means, there are the general best methods of determination which, for each ion, depend on its properties, concentration range and on the particular matrix.

These electroanalytical techniques complemented with spectrophotometry provided means to evaluate the cellular uptake of iron, chromium, nickel as well as total and
ionised calcium and phosphorus (cells were exposed to stainless steel corrosion products or to the corresponding separate major metal ions) and associated with morphological (observation by light microscopy, scanning electron microscopy coupled with X-ray microanalysis as well as transmission electron microscopy), histochemical (identification of alkaline phosphatase positive cells and calcium and phosphates deposits) and biochemical (enzymatic reduction of MTT, alkaline phosphatase activity, DNA content and total protein content) assays to shed some light on the dose- and time-effects of the degradation products on rabbit, rat and human osteoblast-like cells morphology, proliferation, differentiation and function.

The first in vitro biocompatibility test performed in this study was established using bone marrow from rabbit origin, cultured in experimental conditions reported to favour osteoblastic differentiation namely, in the presence of 2.52x10^-4 mol/L ascorbic acid, 1.00x10^-2 mol/L β-glycerophosphate and 10^-8 mol /L dexamethasone. Results revealed that control cultures presented osteoblastic features, as demonstrated by the high activity of alkaline phosphatase and formation of mineralised nodules and also, that 316L stainless steel corrosion products and the corresponding separate major metal ions may affect the expression of the osteogenic phenotype. Furthermore, this simple assay (only MTT reduction, alkaline phosphatase activity and histochemical assays were evaluated) helped to define the more interesting degradation products concentration range to investigate and, served, mainly to provide samples for the optimisation of the analytical processes (digestion, electroanalytical and spectrophotometric procedures) related to the evaluation of the cell culture medium concentration changes in iron, chromium, nickel, total and ionised calcium and phosphorus.
The second biocompatibility assay was performed using rat bone marrow derived cells and taken together, the results of the first two assays, which focused particularly on the relationship between dose of degradation products and its effect on osteoblastic cells, seemed to point out that rabbit cells are less sensitive to metal ions probably because their higher metabolic rate (remember the formation of the mass of roll tissue by day 14 in all rabbit cultures) and also that, generally, the severity of the cellular response increases as the concentration of the metallic slurries at which cells are exposed increases.

Bearing in mind that bone tissue in vivo is probably not exposed to concentrations that cause cell death and based upon our results which demonstrated that 0.100% (i.e. $8.95 \times 10^{-6}$ mol/L Fe $+ 2.35 \times 10^{-6}$ mol/L Cr $+ 1.72 \times 10^{-6}$ mol/L Ni or $8.78 \times 10^{-6}$ mol/L Fe $+ 4.31 \times 10^{-6}$ mol/L Cr $+ 2.56 \times 10^{-6}$ mol/L Ni) of the electrochemically prepared stainless steel slurries disturbed significantly the normal behaviour of osteoblast-like cells, the effects promoted by this sublethal concentration on rat and human (whenever possible to use, this in vitro model is the more advisable since it is the closer to the clinical situation) osteoblast-like cells morphology, proliferation and function, as well as its influence on the differential response of cell populations representative of human osteoblastic cells in different stages of differentiation were extensively investigated. Interestingly, nickel and chromium (not the species in the trivalent state) were found to be retained by rat and human cells and appear to be the elements responsible for the toxicity of stainless steel corrosion products at the concentration studied.
Further investigations focusing on detailed characterisation of degradation products as well as their cellular uptake and storage are therefore needed to complement the data presented in this work, which indicate that corrosion products released into the surrounding tissues by stainless steel implants may markedly influence the fate of the bone-implant interface and the implant survival.

REFERENCES


IV- APPENDIXES
APPENDIX A

Optimised programs used in electrothermal atomic absorption spectrometry for iron, chromium and nickel determinations in cell culture medium digests.

Table A1- Program for iron determination

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Int. Flow</th>
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<tr>
<td></td>
<td></td>
<td>Ramp</td>
<td>Hold</td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>20</td>
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<td>2</td>
<td>140</td>
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<td>30</td>
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<td>3</td>
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<td>4</td>
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<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2400</td>
<td>1</td>
<td>2</td>
<td>250</td>
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</table>

Pipet Speed: 100%

Table A2- Program for chromium determination

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<th>Temperature (°C)</th>
<th>Time (s)</th>
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<th>Read</th>
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</thead>
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<td></td>
<td></td>
<td>Ramp</td>
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</tr>
<tr>
<td>1</td>
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<td>30</td>
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</tr>
<tr>
<td>3</td>
<td>1500</td>
<td>10</td>
<td>20</td>
<td>250</td>
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<tr>
<td>4</td>
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<td>0</td>
<td>5</td>
<td>0</td>
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<tr>
<td>5</td>
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<td>1</td>
<td>2</td>
<td>250</td>
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</tbody>
</table>

Pipet Speed: 100%

Table A3- Program for nickel determination

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<th>Step</th>
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<th>Time (s)</th>
<th>Int. Flow</th>
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<td>Ramp</td>
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</tr>
<tr>
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<td>110</td>
<td>1</td>
<td>20</td>
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<td>250</td>
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<td>3</td>
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<tr>
<td>5</td>
<td>2500</td>
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<td>2</td>
<td>250</td>
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Pipet Speed: 100%

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APPENDIX B

Composition of α- Minimum Essential Medium (Table B1), Hank’s Balanced Salt Solution (Table B2) and Dulbecco’s Phosphate Buffer Solution (Table B3).
Table B1 - Composition of α- Minimum Essential Medium (α-MEM; Sigma M 0894)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (g/L)</th>
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</thead>
<tbody>
<tr>
<td><strong>Inorganic salts</strong></td>
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</tr>
<tr>
<td>Calcium chloride.2H₂O</td>
<td>0.265</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.09767</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.400</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6.800</td>
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<tr>
<td>Sodium phosphate monobasic</td>
<td>0.122</td>
</tr>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
</tr>
<tr>
<td>L- Alanine</td>
<td>0.025</td>
</tr>
<tr>
<td>L- Arginine.HCl</td>
<td>0.12664</td>
</tr>
<tr>
<td>L- Asparagine.H₂O</td>
<td>0.050</td>
</tr>
<tr>
<td>L- Aspartic acid</td>
<td>0.030</td>
</tr>
<tr>
<td>L- Cysteine.HCl.H₂O</td>
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<tr>
<td>L- Cystine.2HCl</td>
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</tr>
<tr>
<td>L- Glutamic acid</td>
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<td>L- Glutamine</td>
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</tr>
<tr>
<td>Glycine</td>
<td>0.050</td>
</tr>
<tr>
<td>L- Histidine.HCl.H₂O</td>
<td>0.042</td>
</tr>
<tr>
<td>L- Isoleucine</td>
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</tr>
<tr>
<td>L- Leucine</td>
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<tr>
<td>L- Lysine.HCl</td>
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<tr>
<td>L- Methionine</td>
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<tr>
<td>L- Phenylalanine</td>
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<tr>
<td>L- Proline</td>
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<td>L- Serine</td>
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<tr>
<td>L- Threonine</td>
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<tr>
<td>L- Tryptophan</td>
<td>0.010</td>
</tr>
<tr>
<td>L- Tyrosine 2Na.2H₂O</td>
<td>0.0519</td>
</tr>
<tr>
<td>L- Valine</td>
<td>0.046</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
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<td>L- Ascorbic acid</td>
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<tr>
<td>Biotin</td>
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<td>Choline chloride</td>
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<td>Folic acid</td>
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<td>Niacinamide</td>
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<tr>
<td>o-Pantothenic acid (hemicalcium)</td>
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<tr>
<td>Pyridoxal.HCl</td>
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<td>Phenol red (sodium)</td>
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<td>pH at 25°C (with sodium bicarbonate)</td>
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### Table B.2- Composition of Hank’s Balanced Salt Solution (HBSS; Sigma H 8284)

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<thead>
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<th>Components</th>
<th>Concentration (g/L)</th>
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<tr>
<td><strong>Inorganic salts</strong></td>
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<tr>
<td>Calcium chloride.2H₂O</td>
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<td>Sodium bicarbonate</td>
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</tr>
<tr>
<td>Sodium phosphate dibasic</td>
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<tr>
<td><strong>Other</strong></td>
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<tr>
<td>Glucose</td>
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<td><strong>Specifications</strong></td>
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<tr>
<td>pH at 25°C (with sodium bicarbonate)</td>
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### Table B.3- Composition of Dulbecco’s Phosphate Buffer Solution (PBS; Sigma D 1408)

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<th>Components</th>
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</tr>
<tr>
<td>Sodium chloride</td>
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<td>Sodium phosphate dibasic.7H₂O</td>
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