



Biodegradable FeMn Bone Graft Substitutes: *In Vitro* and *In Vivo* Testing

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**Submitted in partial fulfilment of the requirements of the Degree of Master of Science in
Anatomy**

March 2023



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Abstract

Metallic biodegradable bone graft substitutes are still in the early stages of development. Nonetheless, they promise to provide a solution to taxing orthopaedic scenarios involving bone loss. To this end, they must be able to balance corrosion rate, cytotoxicity and structural support. Once *in vivo*, the ideal implant must be able to provide mechanical support in load-bearing orthopaedic scenarios, whilst also allowing newly-forming bone to replace it. Biodegradable Fe-based alloys are currently under study in this respect as they provide desirable structural characteristics. However as yet, the ideal balance between corrosion rate and cytotoxicity has not been established.

In this project, pure Fe, Fe35Mn, Fe35Mn1Ag and Fe35Mn5Ag alloys in both as-ground and pre-corroded states were tested for *in vitro* cytotoxicity using direct contact techniques. They were found to cause significant cytotoxicity to two bone cell lines with no viable hFOB (as-ground) or MC3T3-E1 (pre-corroded) cells remaining after a four-day test period.

In vivo experimentation on rats was also performed using Fe35Mn, Fe35Mn1Ag and Fe35Mn5Ag with no cytotoxic effects having been observed throughout a six-month implantation period. All of the implants were well-incorporated into surrounding bone and subsequent histological examination revealed well-formed bone matrix. The only difference between native bone and the newly-formed bone matrix was a black discolouration of the newly-formed bone, likely due to the presence of Mn corrosion products. While favourable biocompatibility results were obtained, the implants only exhibited very minimal corrosion throughout the six-month implantation period. It is likely that the slow corrosion may have contributed to these materials' biocompatibility, suggesting that longer periods of pre-corrosion *in vitro* may yield more favourable cytotoxicity results.

This work demonstrates the inherently cytotoxic nature of Fe alloys. Future work and development will need to focus on accelerating their rate of corrosion while maintaining their structural qualities. All the while, this must be done without increasing their cytotoxicity.

Acknowledgements

I would like to express my gratitude towards my supervisors, Prof. Pierre Schembri Wismayer (Department of Anatomy, Faculty of Medicine and Surgery) and Prof. Ing. Joseph Buhagiar (Department of Metallurgy and Materials Engineering, Faculty of Engineering). They have guided me throughout this project and were instrumental in seeing it to conclusion.

I would also like to extend my thanks to the Tissue Culture Laboratory team, whom I have inundated with questions on several occasions. Their wealth of experience has been a constant guide and has ensured the achievement of the best possible results.

Thanks also goes to the team at the Metallurgy and Materials Engineering Department, in particular Ms. Christabelle Tonna. This dedicated group of individuals has been a crucial source of knowledge and guidance with respect to the engineering side of this project.

A special thank you to my wife Deborah and family, whose constant support and encouragement have been vital. Without their help, this process would have been infinitely more difficult.

My gratitude also goes to the University of Malta who has provided me with the opportunity to further my studies through this project.

A final thank you goes to the Malta Council for Science and Technology for having funded the BIOSA project, of which this dissertation is part. The BIOSA project (R&I-2017-037-T0) was funded through the FUSION: R&I Technology Development programme.

Submissions for Publication

The following manuscripts have been produced in relation to this project:

TONNA, C., SALIBA, 2019. Iron and its Alloys for Bone Regeneration Scaffolds – A Review.

Xjenza Online, 7, pp.49-64.

SALIBA L., SAMMUT K., TONNA, C., PAVLI, F., VALDRAMIDIS, V., GATT, R., GIORDMAINA, R.,

CAMILLERI, L., ATANASIO, W., BUHAGIAR, J., SCHEMBRI WISMAYER, P., 2023. FeMn

and FeMnAg Biodegradable Alloys: An *In Vitro* and *In Vivo* Investigation. – Submitted

to *Heliyon* for consideration for publication.

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List of Abbreviations

Ag	Silver	DMSO	Dimethyl sulfoxide
Al	Aluminium	DMT-1	Divalent metal transporter-1
AP	Alkaline phosphatase	ECV304	Human umbilical vein endothelial cell line
ATP	Adenosine triphosphate	EDS	Energy dispersive X-ray spectroscopy
BCC	Body-centred cubic	FBS	Foetal bovine serum
BMP	Bone morphogenetic protein	FCC	Face-centred cubic
BMU	Basic Multicellular Unit	Fe	Iron
C	Carbon	FREC	Faculty Research Ethics Committee
Ca	Calcium	H	Hydrogen
CaCO ₃	Calcium carbonate	H ₂ O	Water
Ca ₃ (PO ₄) ₂	Calcium phosphate	HA	Hydroxyapatite
Cl	Chlorine	HCl	Hydrochloric acid
Co	Cobalt	hFOB	Human foetal osteoblast cell line
CO ₂	Carbon dioxide	HIV	Human immunodeficiency virus
CO ₃ ²⁻	Carbonate	HLA	Human leukocyte antigen
Cr	Chromium	HMTA	Hexamethylenetetramine
CT	Computed tomography		
Cu	Copper		
DBM	Demineralised bone matrix		
DMEM	Dulbecco's Modified Eagle Medium		

HTLV	Human T-lymphotropic virus	MRI	Magnetic resonance
HUVEC	Human umbilical vein endothelial cell		imaging
HA	Hydroxyapatite $(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)$	MSC	Mesenchymal stem cell
IGF	Insulin-like growth factor	MTT	3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide
IPA	Isopropyl alcohol	N	Nitrogen
JFARRS	Joint Faculty Research Ethics Committee Animal Research Sectoral Subcommittee	Na	Sodium
K	Potassium	NCP	Non-collagenous protein
L929	Murine fibroblast cell line	Nd	Neodymium
MC3T3-E1	Murine pre-osteoblast cell line	Neo	Neomycin resistance gene
MCO ₃	Metal carbonate	Ni	Nickel
MEM	Minimum essential medium	O	Oxygen
MOOH	Metal hydroxide	OPG	Osteoprotegerin
Mg	Magnesium	ORIF	Open Reduction and Internal Fixation
MG63	Human osteosarcoma cell line	P	Phosphorus
Mn	Manganese	PBS	Phosphate buffered solution
MnCl ₂	Manganese chloride	Pd	Palladium
MnO	Manganese oxide	PO ₄ ³⁻	Phosphate
Mo	Molybdenum	PTH	Parathyroid hormone
		RANK	Receptor activator of nuclear factor kappa B

RANKL	Receptor activator of nuclear factor kappa B Ligand	SV40	Simian virus 40
		Ta	Tantalum
		Ti	Titanium
ROS	Reactive oxygen species	TCP	Tricalcium phosphate
S	Sulfur	TGF- β	Transforming growth factor- β
SE	Secondary electron		
SEM	Scanning electron microscopy	WE43	Magnesium alloy, Mg-3.5Y- 2.3Nd-0.5Zr
Si	Silicon	XRD	X-Ray Diffraction
SiC	Silicon carbide	Y	Yttrium
SS316L	Stainless steel, low carbon	Zn	Zinc
SS316LVM	Stainless steel, low carbon, vacuum melted	Zr	Zirconium

Introduction

1.1. Basics of Bone Biology and Healing

Bone is vital to human existence. It serves a number of functions ranging from the protection of vital organs such as the heart and lungs to serving as a store for essential minerals such as calcium (Ca) and phosphorus (P). It also contains bone marrow within it, a tissue with essential functions in haematopoiesis (Grabowski 2009). Perhaps the most interesting of its functions relating to the field of orthopaedics is locomotion, since it provides a basis to which muscles, tendons and ligaments can attach in order to bring about the movement and orientation of the human body in space.

Despite bone's gross appearance as stiff and static, it is actually a complex and dynamic tissue. Multiple cell types are involved in its constant turnover, remodelling and repair, enabling it to heal through the formation of new bone. This is a capability not shared by most other human tissues such as skin, which will instead tend to heal by the formation of fibrotic scar tissue (Datta, Ng et al. 2008, Florencio-Silva, Sasso et al. 2015, Reinke and Sorg 2012, Sorg, Tilkorn et al. 2017). The way bone heals is of particular interest to modern orthopaedic practice as all forms of management of a fracture, whether operative or non-operative, are based on a bone's ability to heal itself through the formation of new bone.

In order to carry out this function, bone employs one of two healing methods; direct (primary) or indirect (secondary) bone healing, with the route chosen depending very much on the mechanics and biology of the fracture site (Ghiasi, Chen et al. 2017, Kim, See et al. 2020, Marsell and Einhorn 2011, Remedios 1999). Primary bone healing occurs in situations of high stability where matching, unfragmented bone ends are brought and kept together by

means of an operative procedure. In situations where this is not possible, indirect bone healing occurs. Here, multipotent mesenchymal stem cells (MSC) become essential, with these cells being able to commit to a lineage of bone forming cells (Bianco 2014, Wang, Wang et al. 2013).

Even though bone's healing potential is tremendous, this capability still has its limits. Certain injuries or mechanisms of injury such as open tibial fractures and high-velocity motor vehicle accidents have the capacity to create larger defects known as critically-sized bone defects. These injuries exhaust the healing capabilities of bone and must therefore be dealt with differently. In this regard, bone grafting techniques occupy a very important niche. (Adamczyk, Meulenkamp et al. 2020, Keating, Simpson et al. 2005, Nauth, Schemitsch et al. 2018).

1.2. Bone Grafts

In addition to their use in trauma, bone grafts have also been employed in other areas such as fracture non-union and revision major joint arthroplasty surgery. This is due to their ability to fill bone defects and also for their potential to alter the local bone biology, promoting bone formation and ultimately, healing (Choi, Bae et al. 2021, Gamradt and Lieberman 2004, Lee, Son et al. 2021, Lei, Hu et al. 2019).

The traditional bone grafts are the autograft and the allograft. Autologous bone grafts, also referred to as autografts, employ bone harvested and implanted within the same individual. This form of graft is typically heralded as the gold standard graft and is generally the most easily and readily available (Miller and Chiodo 2016, Schmidt 2021). The establishment of bone banks however, has since allowed for the ability to use bone allografts more easily and these are now a well-founded alternative to autografts. This form of graft employs bone

harvested from a separate, most often deceased, donor (Baldwin, Li et al. 2019). Bone banks can however be difficult and expensive to run, making their establishment in certain regions difficult (de Alencar and Vieira 2010). As a result, many countries have yet to provide such a service or are only just beginning to do so (Ali, Mubarak et al. 2022).

At present, autografts and allografts are undoubtedly the mainstay of bone grafting, although each presents its own inherent problems. Autologous bone grafts necessitate the creation of a separate wound within the same patient, which brings with it the risks of associated pain, potential for infection and may also increase the duration of the procedure. The supply of autografted bone is also limited and this option may not always be viable in patients with multiple, severe injuries (Attia, Mahmoud et al. 2021, Myeroff and Archdeacon 2011).

Allografts may also be limited in availability and in addition, have the potential to transmit both bacterial and viral infections such as *Staphylococcus aureus*, Human Immunodeficiency Virus (HIV) and human T-Lymphotropic Virus (HTLV). Manipulation, sterilisation and storage of the harvested bone in order to decrease these infection risks and render them available over extended periods of time may also affect their function with regards to biological milieu (Fishman, Greenwald et al. 2012, Zamborsky, Svec et al. 2016).

1.3. Bone Graft Substitutes

In response to the issues encountered with bone autografts and allografts, the scientific community has begun to look for alternatives, hence the development of bone graft substitutes. This third group of bone grafts has so far included a number of biological and man-made substances. These grafts have the potential to replace the traditional autograft and allograft and therefore remove their potential complications (Finkemeier 2002, Laurencin,

Khan et al. 2006). However, in order for them to function correctly, they must at least possess one of the following qualities; (1) osteoconduction; (2) osteoinduction and (3) osteogenesis (Albrektsson and Johansson 2001, Giannoudis, Dinopoulos et al. 2005).

Osteoconduction is the ability of a graft to allow newly-forming bone to grow through its structure, necessitating an element of porosity. Osteoinduction on the other hand is the ability of a graft to stimulate the differentiation of undifferentiated, multipotent cells such as MSC along a bone-forming cell lineage. Finally, osteogenesis is the ability of the graft to directly produce bone itself and is most frequently the result of bone-forming cells found within the graft. As a result, this is an ability most frequently exhibited by autografts, which transport within them osteoblasts and MSC. Different from osteoconduction, osteogenesis makes no reference to the passage of bone through the graft but is instead the graft's innate ability to produce bone.

The ceramic biomaterial β -tricalcium phosphate (β -TCP) is one of the most widely used bone graft substitute materials and is often available in the form of granules or wedges. This material is very well known to be osteoconductive but has also more recently been shown to possess some osteoinductive properties (Bohner, Santoni et al. 2020, Gao, Zhang et al. 2016). This combination of properties has ensured that β -TCP fills an important niche in current orthopaedic practice. Other bone graft substitutes such as those containing bone morphogenetic proteins (BMP) employ osteoinduction (Oryan, Alidadi et al. 2014).

In spite of these desirable characteristics, the currently available bone graft substitutes tend to fail at an important stumbling block; they lack the mechanical properties necessary for such implants to perform adequately in load-bearing orthopaedic applications (Haugen, Lyngstadaas et al. 2019, Lobb, DeGeorge et al. 2019).

Porous Titanium (Ti) has been one proposition in response to this problem. This material is known to have desirable mechanical characteristics, even when employed as a porous scaffold. That being so, it has been developed with the fields of orthopaedics and dentistry in mind (Dabrowski, Swieszkowski et al. 2010, Oh, Nomura et al. 2003, Pałka and Pokrowiecki 2018, Wally, Van Grunsven et al. 2015)

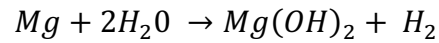
1.4. Metal Bone Graft Substitutes

Ti is a promising material, however it is imperfect. Although it provides the graft with valuable mechanical characteristics, it is not a bioresorbable material, which is in fact a major reason as to why it is used in implants such as hip arthroplasty prostheses (Malahias, Kostretzis et al. 2020). While it would be beneficial for such implants to remain uncorroded, Ti's corrosion resistance may compromise its ability to be used as a bone graft substitute material.

An attempt at solving this crucial issue has thrust corroding metal bone graft substitutes into the limelight. In addition to their promising mechanical attributes, certain metals are able to corrode over time, as bone growth and healing continue. The ideal situation would eventually see the graft entirely replaced by newly-formed bone. Magnesium (Mg) was an early proposition as it promised to provide a biocompatible implant (Agarwal, Curtin et al. 2016, Kamrani and Fleck 2019, Li, Gao et al. 2004, Maguire and Cowan 2002, Song 2007). While possessing the ability to degrade, Mg-based implants were shown to corrode at a rate too rapid to allow meaningful bone formation within the graft, once again leaving behind a bone defect. This effect was most noticeable in Chloride (Cl) rich environments, an ion which is abundant within the normal human body's biochemical milieu (Agarwal, Curtin et al. 2016, Kamrani and Fleck 2019, Kraus, Fischerauer et al. 2012, Lu, Deshmukh et al. 2021, Song 2007, Tsakiris, Tardei et al. 2021, Wang and Shi 2011, Witte, Fischer et al. 2006). The reaction may

lead to the release of significant amounts of Hydrogen (H₂) gas as described in Equation 1, and it is thought that this may give rise to tissue injury (Heiden, Walker et al. 2015).

Equation 1 – The reaction between Mg and H₂O, generating H₂ gas.



Because of these limitations, Mg-based implants have found potential applications in other fields of medicine, most notably as cardiovascular stents (Haude, Erbel et al. 2016, Heublein, Rohde et al. 2003, Li, Wang et al. 2019). Since then, other metals such as Tantalum (Ta) have also come under scrutiny but these too have downfalls. Thus far, these deficiencies have rendered them inadequate for the purposes of a corroding bone graft substitute (Bohner 2010).

Iron (Fe) was proposed as a potential material in this regard. Fe and its alloys have shown potential with regards to a mechanically desirable, biodegradable and biocompatible implant. As a physiologically acceptable element, Fe can be alloyed with other elements such as Manganese (Mn) and Silver (Ag). Mn is of particular relevance in this case as it allows the transformation of the body-centred ferrite phase to the face-centred austenitic phase of the alloy at a particular temperature, through a process known as austenisation (Kempen, Sommer et al. 2002). This process also ensures that the new alloy is anti-ferromagnetic, allowing compatibility with modern and commonly used medical imaging techniques such as magnetic resonance imaging (MRI). In addition, the corrosion rate of these materials is slower than that of Mg, allowing more time for new bone to form (Čapek, Kubásek et al. 2016, Hermawan, Dubé et al. 2010, Liu, He et al. 2019, Liu, He et al. 2018, Venezuela and Dargusch

2020). Different alloys of these elements may therefore allow the manufacture of a corroding metal bone graft substitute that is osteoconductive through the regulation of pore size within the structure, along with mechanical strength and potentially even bacterial resistance (Sotoudehbagha, Sheibani et al. 2018).

The promise that FeMnAg alloys hold however comes at a potential biological cost. As these alloys corrode, they release potentially toxic corrosion products into their surrounding environment. Therefore, a balance must be struck between corrosion rate and biocompatibility. The faster the corrosion, the greater the likelihood of cytotoxicity and vice versa.

1.5. Cytotoxicity and Biocompatibility

In order to understand the potential of Fe alloys to perform in their role as bone graft substitutes, it is necessary to perform both *in vitro* and *in vivo* tests. The *ISO 10993-5:2009, Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity* outlines methods for both direct and indirect contact cytotoxicity testing. While there is a paucity of evidence targeting FeMn-based alloys, the little *in vitro* evidence that is available is mostly performed using indirect cytotoxicity testing techniques (Čapek, Kubásek et al. 2016, Dargusch, Dehghan-Manshadi et al. 2019). While this represents a valid testing method, it is felt that this technique does not accurately reproduce the increasing corrosion products that may form *in vivo*, with their potential to increase cytotoxicity. This would likely be more accurately represented using direct contact testing methods where cells are grown in direct contact with the metal implants themselves.

Even less evidence is available with regards to *in vivo* biocompatibility, especially when considering the requirement for the austenitic phase of FeMn (Drynda, Hassel et al. 2015).

With this, it becomes clear that there exists a lacuna in knowledge regarding the *in vitro* and *in vivo* cytotoxicity and biocompatibility performance of FeMn and FeMn-based corroding bone graft substitutes. This lacuna is the focal point that this project targets.

1.6. Cell Viability Assays

The understanding of cell viability is crucial to the study of *in vitro* cytotoxicity of materials. Many assays are currently available, each working differently and with its own advantages and disadvantages.

The MTT assay is one of the most commonly used assays and relies on the reduction of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), to the purple chemical formazan. This is only possible in metabolically active cells which contain NAD(P)H-oxidoreductase enzymes (Meerlo, Kaspers et al. 2011, Mosmann 1983, Plumb 2004). The problem with the MTT assay with regards to this project is that it is a redox-based test and so there is a potential for Fe in the samples to interfere with this process and produce spurious, unreliable results (Habtemariam 1995).

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) assays are also commonly used, with PrestoBlue™ and AlamarBlue™ being two examples. Similar to the MTT assay, these cell viability assays also rely on metabolically active cells to reduce resazurin to resorufin (7-hydroxy-3H-phenoxazin-3-one), with an accompanying change in colour from blue to red. Although easy and quick to use, resazurin assays are redox-based and therefore they too run the risk for interference by Fe (O'Brien, Wilson et al. 2000).

The CellTiter-Glo® assay works through the function of the luciferase enzyme, which generates a luminescent signal based on the amount of adenosine triphosphate (ATP) present in the test sample. This assay also contains inhibitors of ATPases, important enzymes which

mop up any ATP lost into the surrounding environment after cell death. The reaction is quick and generates a stable luminescent signal which can increase linearly over several logarithmic scales, depending on the number of viable cells in the test sample (Hannah, Beck et al. 2001, Riss, Moravec et al. 2016). Such assays have been shown to work reliably in several situations, including where viability must be tested in the presence of a microporous structure (Braun, Stürzel et al. 2018). An essential factor to this project is the fact that this assay is not redox based and therefore should not be affected by the presence of Fe in the test samples. For these reasons, CellTiter-Glo® was selected as the assay for cytotoxicity testing in the *in vitro* portion of this project.

1.7. Aims and Objectives

1.7.1. Aim

Biodegradable metal bone graft substitutes represent a relatively new field of research. As a result, little is known about their potential to fulfil this role. While Fe-based materials promise to provide mechanically desirable, bioresorbable grafts, there is very little evidence referencing FeMn and FeMnAg's *in vitro* cytotoxicity and *in vivo* biocompatibility.

This project is aimed squarely at this gap in knowledge and is designed to employ both *in vitro* and *in vivo* testing methods to better understand the potential of FeMn-based alloys as materials for corroding metal bone graft substitutes.

1.7.2. Research Questions

Do FeMn-based materials assume the qualities necessary for bioresorbable bone graft substitutes? Specifically, do they cause cytotoxicity to an extent which would render them

non-biocompatible and do they corrode at a rate which is adequate to allow for repair of bone defects?

1.7.3.Objectives

In order to answer this project's research questions, the relevant materials must be tested in both *in vitro* and *in vivo* situations. To this effect, the following objectives have been set:

1. To manufacture pure Fe and austenitic FeMn-based coupons and pins through a process of powder metallurgy. Subsequent characterisation will document their composition and crystalline structure.
2. To understand the cytotoxic profile of the resulting alloys, *in vitro* cytotoxicity testing will be performed where cell viability will be measured after direct exposure to these materials. This process will be performed twice in order to increase reproducibility and reliability, especially considering that *in vivo* tests will be performed on rats and not humans. A biological replicate will also allow for the study of the effect of pre-corrosion of these materials on cell viability.
3. To perform an *in vivo* experiment where FeMn-based pins will be implanted into the vertebrae of rats. Examination of the incorporation and corrosion of the *in vivo* implants after a six-month period, along with histological examination of surrounding bone will help to gain an understanding of any negative effects of corrosion.

Literature Review

2.1. Bone Biology

Bone is a complex human tissue with different components working together to provide its important functions. These range from protection of vital internal organs, to locomotion and the storage of essential minerals such as Ca and P (Grabowski 2009). At its most basic level, bone consists of two major parts, these being the cellular and matrix components. Bone's matrix is the larger component by a large margin, occupying approximately 90% of healthy, mineralised adult bone. The smaller cellular component forms the remaining 10% of bone and is essential to its health as it retains the ability to control its formation, degradation, turnover and remodelling. The interaction between these two components is vital and allows bone to achieve all of its functions, perhaps the most notable of which is movement of the human body (Miller and Thompson 2019, Quarles 2008, Ramachandran 2017).

2.1.1. Cellular Components of Bone

In health, bone is seen to contain three main cellular types. These are the osteocyte, osteoblast and the osteoclast. Each of these cell types has distinct and varied functions but it is the interactions between these cells that allows bone to function as a dynamic tissue, whether this be remodelling, fracture healing or the regulation of plasma levels of Ca and P (Fattore, Teti et al. 2012).

2.1.1.1. The Osteocyte

The osteocyte is the most abundant cell within normal, healthy bone and comprises approximately 90% of its cellular population. This cell is a former osteoblast which has become

entrapped and surrounded by mineralised bone matrix. It therefore shares cell lineage with osteoblasts, originating from mesenchymal stem cells (MSC) (Noble 2008). Osteocytes are found within both woven and lamellar bone (Hernandez, Majeska et al. 2004) and are able to communicate with each other via cell processes running through conduits within the bone called canaliculi, as in Figure 1.

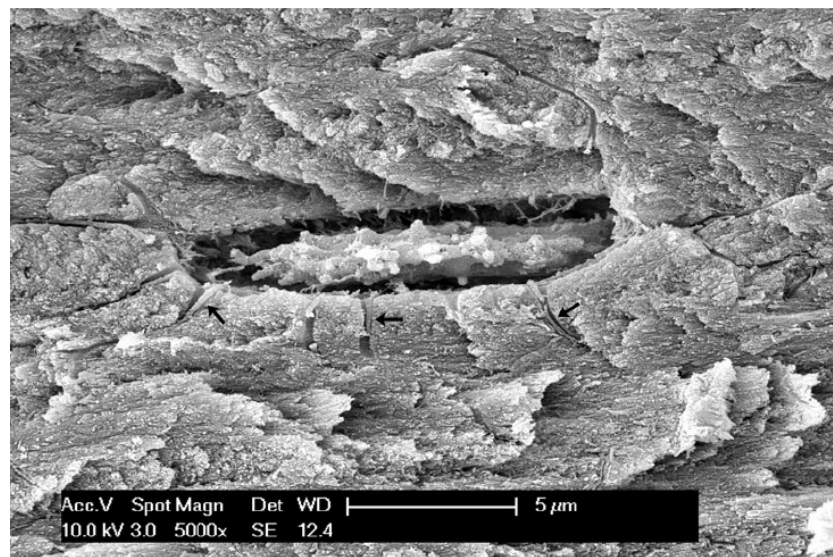


Figure 1 – Scanning electron microscopy image of an osteocyte. It is encased within bone and communicates with other similar cells via processes (arrows) running within canaliculi. This forms a network within bone that helps osteocytes exert their function. Adapted from Pazzaglia, Congiu et al. (2010) (Pazzaglia, Congiu et al. 2010).

The osteocytes' encasement within bone matrix may suggest that they are not particularly active. While it is true that they are not as directly involved as osteoblasts in the laying down of new bone, they are in fact prolific with regards to the regulation of bone mass, remodelling and the regulation of essential plasma minerals such as Ca and P. Needless to say, osteocytes are an essential component of the dynamic nature of this tissue.

Osteocytes are for example sensitive to hormones such as calcitonin which activates them, and parathyroid hormone (PTH) which inhibits them (Bellido, Saini et al. 2013). As an

important part of, and in addition to their endocrine function, osteocytes are able to regulate and control the formation and degradation of bone. They exert this function through their sensitivity not only to signalling molecules but also to mechanical stimuli, which trigger them to coordinate the function of both osteoblasts and osteoclasts. These cells are the primary bone forming and degrading (respectively) cells which are also located here (Robling and Bonewald 2020).

The Sost/Wnt signalling pathway is one of the best understood pathways through which osteocytes exert regulatory control over osteoblasts. The sclerostin (Sost) gene is located on chromosome 17 (Brunkow, Gardner et al. 2001, Sebastian and Loots 2018) and is responsible for the production of sclerostin protein within osteocytes. When released, sclerostin causes a decrease in signalling through a group of pathways known as the Wnt signalling pathways. Ultimately, the result of upregulated Sost function (and therefore of the protein sclerostin) is to bring about an inhibitory effect on osteoblast-led bone formation (Delgado-Calle, Sato et al. 2017, Krishnan, Bryant et al. 2006, Moester, Papapoulos et al. 2010, Yavropoulou and Yovos 2007). Abnormalities of this pathway have been documented to be present in several disorders of bone mass. One such disorder is sclerosteosis, where a mutation affecting the Sost gene causes decreased Sost and sclerostin function with resultant abnormal increase in bone mass (Brunkow, Gardner et al. 2001, Semënov, Tamai et al. 2005, van Lierop, Appelman-Dijkstra et al. 2017).

2.1.1.2. The Osteoblast

In health, osteoblasts are present in much smaller numbers than osteocytes, with which they share MSC lineage (Long 2012). This cell type is key to the formation of new bone, being responsible for both the formation of bone matrix and its subsequent mineralisation. In doing

so, osteoblasts play a central role in the normal turnover and remodelling of bone (Eriksen 2010, Hadjidakis and Androulakis 2006). They take centre stage in certain pathologies such as trauma and fracture healing, where they interact with other cell types and modulators in order to unite fracture fragments (Miller and Thompson 2019, Ramachandran 2017).

Regulation of osteoblast function is a complex affair and a balance between their function and the function of other cells such as osteoclasts is necessary for normal bone biology. To this effect, hormones such as parathyroid hormone (PTH) and vitamin D play an essential role. These, alongside other cytokine and cellular messenger molecules such as transforming growth factor- β (TGF- β), Insulin-like growth factor (IGF) and bone morphogenetic proteins (BMP), play an essential role in the regulation of osteoblast function (Aubin 2001, Harada and Rodan 2003, Wu, Chen et al. 2016).

Once activated, osteoblasts first begin to lay down unmineralized bone matrix. They achieve this through the production and deposition of collagen. This protein is laid down on an existing bone surface known as the mineralisation front, with type I collagen being the most commonly found within bone. This unmineralized, organic matrix is subsequently mineralised, predominantly through the deposition of calcium hydroxyapatite (HA), $(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2)$. Once again, this function is carried out by osteoblasts which, with the aid of regulatory proteins such as osteocalcin and osteonectin, are able to deposit calcium HA (among other minerals), in a highly ordered manner (Caetano-Lopes, Canhao et al. 2007, Miller and Thompson 2019, Ramachandran 2017).

In addition to their undeniably important role in bone formation, osteoblasts also function to regulate the activation and function of osteoclasts, the final major component of the cellular aspect of bone.

2.1.1.3. The Osteoclast

The osteoclast differs from both osteocytes and osteoblasts in several aspects, beginning with cell lineage. Rather than originating from MSC, osteoclasts are instead derived from a haematopoietic, macrophage cell lineage. In addition, these cells' function is not to form bone, but to degrade it. They do this by resorbing the inorganic mineral component and subsequently breaking down the organic component of bone matrix. This process releases the important minerals Ca and P, allowing osteoclasts to play an important role in their regulation in blood plasma (Blair, Kahn et al. 1986, Miller and Thompson 2019, Ramachandran 2017).

In non-pathological states, osteoclasts are mostly present as inactive pre-osteoclasts. This cell type is susceptible to activation through a number of signalling pathways, in which osteoblasts and osteocytes are often involved. The receptor activator of nuclear factor kappa-B (RANK) pathway is one such important signalling pathway in the activation and control of this cell type. RANK ligand (RANKL) is released by both osteoblasts and osteocytes and binds RANK receptors located on the surface of inactive pre-osteoclasts. This activates them to osteoclasts and triggers the process of bone resorption. To combat and regulate this, osteoblasts also produce a protein known as osteoprotegerin (OPG). This molecule binds free RANKL, preventing it from binding and activating its RANK receptor target on pre-osteoclasts. In turn, this prevents the activation of these cells to osteoclasts (Boyce and Xing 2007, Boyle, Simonet et al. 2003, Lorenzo 2017). This important balance has become the target of pharmacologic agents such as Denosumab which, in cases of malignancy and pathological fracture, are aimed at regulating bone mass and bone mineral density (Castellano, Sepulveda et al. 2011).

Although bone destruction may appear at first glance to be detrimental to bone health, it is contrarily essential. Resorption of bone is one of the facets of normal bone turnover and

remodelling (Soysa and Alles 2016), allowing it to adapt to changing stresses. This is the reason why bone is able to resist fatigue fracture and cope with a gradually increasing workload. Contrarily, disuse osteopenia and similar issues occur when patients restrict loading of a limb and reduce the mechanical stimulus to which the bones are exposed. This in turn reduces bone mass and mineral density, predisposing these patients to fragility fractures (Boyle and Kim 2011, Chen, Liu et al. 2010, Teichtahl, Wluka et al. 2015).

Bone resorption is also an essential component of fracture healing, both by primary and secondary healing processes (Little, Rogers et al. 2011). It is evident therefore that without this resorption, normal function would be impossible. This is true not simply with regards to locomotion, but also for other processes such as the regulation of Ca and P levels within blood plasma.

2.1.1.4. *In Vitro* Cell Lines Used for Cytotoxicity Testing

An understanding of the most common and important cells present in normal human bone has led to the use of a number of cell lines in *in vitro* cytotoxicity testing. Two of the most important are human foetal osteoblasts (hFOB) and murine pre-osteoblast (MC3T3).

2.1.1.4.1. hFOB Cell Line

The hFOB cell line was initially obtained as a primary culture from a spontaneous miscarriage. These cells were then transfected with the Simian virus 40 (SV40) large T antigen, effectively immortalising the cell line when incubated at a permissive temperature of 34°C. Alongside this gene was the transfected the neomycin resistance gene (Neo). The 1.19 clone was then selected and examined for its high expression of alkaline phosphatase (AP). The result was an immortalised and well-differentiated line of osteoblasts with desirable

characteristics for the *in vitro* study of osteoblasts(Bryan and Redder 1994, Harris, Enger et al. 1995).

2.1.1.4.2. MC3T3-E1 Subclone 4 Cell Line

MC3T3-E1 cells were initially obtained from the calvaria of new-born mice. Initial work identified these cells to grow in a fibroblastic state with the capacity to differentiate into both osteoblasts and osteoclasts. In addition, this cell line was able to form calcified bone *in vitro* (Sudo, Kodama et al. 1983). This particular cell line was however found to be phenotypically heterogenous and so further subclone isolation yielded a number of cell lines with more or less differentiation towards an osteoblast line. MC3T3-E1 Subclone 4 was in fact found to be highly differentiated towards an osteoblast cell line and was able to form mineralised bone matrix early on during culture. These characteristics make this cell line ideal for *in vitro* work on osteoblasts(Wang, Christensen et al. 1999).

2.1.2. Bone Matrix

Bone matrix forms the largest component of healthy adult bone by a substantial margin. It is formed, maintained and degraded by the cells present within it and is split at its most basic level into two major components. The largest part of this, accounting for approximately 60% of bone matrix, is the inorganic mineral constituent. The remaining proportion comprises a multitude of organic molecules, of which type I Collagen is the most abundant (Boskey and Robey 2013, Miller and Thompson 2019, Olszta, Cheng et al. 2007, Ramachandran 2017, Safadi, Barbe et al. 2009). Together, the organic and inorganic constituents of bone matrix form a composite material which affords desirable mechanical characteristics (Clarke 2008, Keaveny, Morgan et al. 2004). Importantly, the mineralised constituent increases in

proportion with age, affording different mechanical characteristics in an adult (Boskey and Coleman 2010).

2.1.2.1. Organic Matrix

Bone's organic matrix is essential to its structure, function, and mechanical integrity. To a large degree, this component is composed of collagen, a protein molecule which is extremely abundant in the human body and is found in a multitude of locations, from connective tissues to joints, bone and scar tissue (Shoulders and Raines 2009). It is this component of bone which contributes most to tensile strength (Turner 2006) but in addition, collagen provides a framework for mineralisation, allowing bone to become a composite material with superior mechanical characteristics (Nair, Gautieri et al. 2014, Oosterlaken, Vena et al. 2021, Wiesmann, Meyer et al. 2005).

There exist many types of collagen within the body (Ricard-Blum 2011), although type I collagen is far and away the most prevalent form found in bone's organic matrix (Miller and Thompson 2019, Ramachandran 2017). Type I collagen is synthesised by the ribosomes present on the rough endoplasmic reticulum of osteoblasts. It is initially synthesised as procollagen and is composed of three molecular chains, two of which are alpha-1 chains and one being an alpha-2 chain (Scott, Kent et al. 1980). These chains are then linked together by hydrogen bonds to form a right-handed triple helix protein molecule. This molecule undergoes hydroxylation of proline residues within its primary structure and is then released from the cell where cleavage occurs, leaving collagen. Subsequently, further hydrogen bonding between the triple helices occurs to form quarter-staggered arrays of collagen, yielding collagen fibrils (Miller and Thompson 2019, Ramachandran 2017). Cross-linking is an integral

part of this process, having the ability to affect how bone is mineralised, and therefore also having a bearing on bone's final strength (Garnero 2012, Knott and Bailey 1998).

The collagen fibrils formed during this process are laid down as part of the organic bone matrix, allowing the formation of a highly-ordered structure such as the osteon. This is the basic unit of lamellar, cortical bone. The study of this non-random process has revealed a complex, hierarchical structure with collagen arranged differently and therefore exhibiting different functions based on the region of its deposition within the osteon. Ordered collagen fibrils are longitudinally arranged molecules which function mostly in the resistance of tension and compression (when calcified). On the other hand, loosely arranged fibrils have varying orientation and form a region within which canaliculi are found (Reznikov, Shahar et al. 2014, Varga, Pacureanu et al. 2013).

There are many other molecules which can be found in the organic component of bone matrix but perhaps one of the most heavily studied groups is that of the BMPs. This group of molecules falls under the heading of the TGF- β family of molecules and includes proteins which are known to affect the differentiation, survival and function of bone cells (Brazil, Church et al. 2015, Rosen 2006, Yamaguchi, Ishizuya et al. 1996). In doing so, they play a crucial role in normal bone physiology, an aspect which can be manipulated by science in order to impart desirable characteristics to synthetic bone grafts (Belfrage, Flivik et al. 2011, Giannoudis and Tzioupis 2005).

Non-collagenous proteins (NCP) are also important molecules which form part of the organic matrix of bone. This diverse group of proteins includes molecules such as fibronectin, osteopontin and osteonectin. These proteins have varied functions but are also seen to have important roles in the formation, organisation and structure of the mineralised, inorganic component of bone matrix (Young 2003).

2.1.2.2. Inorganic Matrix

The inorganic matrix forms the largest component of bone as a whole. It is that component of the matrix which is constituted by inorganic materials, most abundantly Ca and P. Its main constituent is calcium hydroxyapatite, however it also contains a number of other similar minerals and compounds, in smaller quantities. Two examples of these are brushite (osteocalcium phosphate) and carbonate (CO_3^{2-}) (Miller and Thompson 2019, Ramachandran 2017).

One of the primary functions of the inorganic matrix is to act as a store for Ca and P. Through regulation of osteoblast and osteoclast function, osteocytes (and their regulators) are able to alter the formation and degradation of bone, along with its mineralisation density. This in turn affects the levels of these minerals within human plasma (Bonucci 2012, Miller and Thompson 2019). It is also this mineralised portion of bone which to a large degree, determines the compressive strength of bone. The organic component of bone, namely type I collagen, in contrast mostly contributes to its tensile strength (Ammann and Rizzoli 2003, Malik and Malik 2015, Miller and Thompson 2019, Ramachandran 2017, Turner 2006).

The mineralisation of osteoid is a tightly regulated process. Specific proteins such as osteopontin and osteocalcin are integral to the ordered deposition of hydroxyapatite within regions known as hole and pore zones of the collagen matrix. This ensures that the final product is a highly ordered structure and is best able to deal with the stresses to which it is exposed and expected to deal with (Bourne, Wheeler-Jones et al. 2021, Poundarik, Boskey et al. 2018, Ramachandran 2017, Rey, Combes et al. 2009, Von Euw, Wang et al. 2019).

2.2. Bone Defects and Bone Grafting

The ability of bone to regenerate itself is impressive but even this power has its limits. The creation of a large bone defect, be it secondary to trauma, malignancy or other pathologies, has the potential to exhaust bone's healing capabilities. When this occurs, adjunctive procedures in the form of bone grafting may be required in order to allow for continuity to be restored. Every year in the United States alone, approximately half a million bone grafting procedures are performed. To put this into perspective, bone grafting procedures are the second most commonly performed transplant procedure, second only to blood transplant (Baldwin, Li et al. 2019, Faour, Dimitriou et al. 2011).

2.2.1. Bone Loss and Non-Union

2.2.1.1. Bone Defects

Bone loss is a common problem encountered in modern day orthopaedic and trauma surgery and may come about as a result of several processes. Some of the most commonly faced are defects arising from high energy open fractures such as in the tibia, which occupies a relatively superficial subcutaneous position in the leg. These forms of injury are challenging in the best of scenarios, not least because they involve an essential weight bearing bone. As a result, they must be dealt with expeditiously if other complications such as infection (which may also cause bone loss) are to be avoided (Keating, Simpson et al. 2005, Laine, Cherkashin et al. 2016, Watson, Anders et al. 1995).

Several other pathologies may also lead to defects within bone. These may once again be related to trauma, as may occur in crush injuries and lower-energy fractures, often involving the distal radius and tibial plateau. These injuries may lead to the crushing of subchondral

bone, creating a depressed fracture with underlying bone loss and consequently, a lack of structural support for the joint surface (Keating, Simpson et al. 2005, Ladd and Pliam 1999, Malizos, Dailiana et al. 2010).

Infection is another very important and challenging cause of bone loss and may also be related to trauma, especially when considering high energy open fractures with significant soft tissue compromise (Oliveira, Gomes et al. 2020). Neoplasms and malignancy are well known causes of bone defects, whether this be related to the pathology itself causing bone lysis and fracture, or surgical intervention resecting pathological bone (Cheng, Shih et al. 2001, Wiese and Pape 2010). Arthroplasty surgery presents a particularly challenging situation where bone loss may be severe, especially in cases of revision arthroplasty. Surgery in such cases usually involves more complex techniques to restore the integrity of bone or to replace it altogether (Cuckler 2004, Huff and Sculco 2007).

2.2.1.2. Critically-Sized Bone Defects

Whilst there is no strict consensus on the exact proportions of a critically-sized bone defect, it is generally accepted as being one large enough in size to preclude healing despite operative stabilisation. These defects therefore necessitate further intervention to augment the local bone's healing potential and often involve some form of bone grafting procedure (Nauth, McKee et al. 2011).

Multiple factors come into play when considering the healing potential of bone, including factors relating to the event itself and the level of energy imparted during the trauma (if traumatic). The higher the energy imparted during the initial injury, the more likely the fracture is to be comminuted or fragmented, with devascularised fragments of bone forming as a result. Open fractures may also result in the loss of these fragments outside of the body

with an associated increase in the risk of infection from exposure to a contaminated external environment. The particular bone affected also has a great bearing on the ability to heal with particular bones tolerating defects less well than others (Adamczyk, Meulenkamp et al. 2020).

The patient's physiological status, including co-morbidities and smoking habits also play a large role in defining the healing potential of a bone defect. In addition, diseases such as diabetes mellitus and peripheral vascular disease as well as the patient's state after the traumatic event may all impact the bone's capacity to heal the defect (Adamczyk, Meulenkamp et al. 2020, Nauth, McKee et al. 2011, Roddy, DeBaun et al. 2018, Schemitsch 2017).

Once a critically-sized bone defect has developed, it must be managed accordingly. If appropriate management is not instituted in a timely fashion, fracture healing potential will be compromised and non-union may occur as a result. This chronic condition may ultimately have marked impacts on the patient's quality of life including chronic pain and loss of function, work and income (Brinker, Hanus et al. 2013, Copuroglu, Calori et al. 2013, Wildemann, Ignatius et al. 2021).

Aseptic non-unions occur in the absence of infection and may be either atrophic or hypertrophic. The hypertrophic variety occurs in the setting of adequate blood supply but inadequate stability. In this case, fracture callus does begin to form as it would normally do in an endochondral fracture healing response, but inadequate stability prevents the forming tissues from adequately bridging the fracture. In turn, the healing process is unable to yield a united, remodelled bone with a lamellar structure, leaving instead only fibrous tissue. Atrophic non-unions on the other hand involve an inadequate vascular supply to the fracture, leaving the fracture ends with a poor healing response. Any metalwork present here as part of the operative management of the fracture will eventually succumb to fatigue failure, as

bone fails to form and will therefore not bear the weight of a mobilising patient. Both of these non-unions may also be present in the setting of sepsis, which further complicates the management of such injuries (Frölke and Patka 2007, Panagiotis 2005).

2.2.2. Traditional Bone Grafting

In response to the management issues encountered with bone defects, modern orthopaedic practice often employs bone grafts. Traditionally, two major forms of graft have been employed, these being the autograft and the allograft. At present, the autograft is currently regarded as the gold standard graft (Miller and Chiodo 2016, Schmidt 2021).

2.2.2.1. The Autograft

When an autograft is to be used, the donor and recipient are the same person, meaning that bone is harvested from one site such as the ilium, and transported to a separate site within the same individual, where the bone defect resides. This is advantageous as it removes the possibility of graft rejection as a result of differences in human leukocyte antigen (HLA) between donor and recipient. In addition, it is the most easily available form of bone graft and is harvested and implanted within the same operative procedure. Autografted bone also eliminates the possibility of transmission of diseases such as HIV (Hill 2010).

Depending on the type of bone and the region from which it is harvested, autografted bone may also provide other benefits, including the possibility of providing structural support. This is employed in several situations such as the open reduction and internal fixation (ORIF) of proximal humeral fractures. Fractures here often result in a significant amount of bone loss, precluding a stable fixation with standard ORIF techniques. As a result, a structural autograft such as a fibular strut may be used to augment the ORIF procedure, providing stability and

improving the chances of fracture healing (Saltzman, Erickson et al. 2016). Some other methods of harvesting autograft such as the reamer irrigator aspirator system, do not take advantage of the structural integrity which may be provided by cortical bone grafts (Cox, Jones et al. 2011).

Certain autografts may also be vascularised if harvested with their feeding vessels. Such grafts are employed in several settings such as in fractures of the scaphoid, which may be notoriously difficult to manage in view of their tenuous blood supply. In these cases, the harvested bone may either be pedicled or free. Pedicled grafts involve the harvesting of bone which remains attached to its original blood supply. Free grafts on the other hand are harvested from a completely different site and vascular anastomoses are then performed to a new feeding vessel (Doi, Oda et al. 2000).

Perhaps one of the most beneficial aspects of an autograft is its inherent biology. Autografted bone brings with it all the important extracellular and cellular factors which are important in the integration and remodelling of the graft. The graft itself, as it does not need to be transported between individuals, does not require processing. This prevents damage and loss of important factors such as BMPs present within the autograft and therefore affords favourable healing characteristics, aiding with the integration of the graft. In addition, autografts may also be osteogenic, having the ability to generate bone of their own accord, thanks to their content of cells with bone-generating potential such as osteoblasts and MSC (Goldberg and Akhavan 2005, Khan, Cammisa Jr et al. 2005, Stevenson 1999).

In spite of its many desirable attributes, the autograft also comes with drawbacks. The first and most obvious of these is that bone needs to be harvested before it can then be implanted. More often than not, this entails a separate incision with all of its associated risks and disadvantages, ranging from increased operative times, to risk of infection, fracture, pain and

numbness at the donor site. All of these complications may be the cause of significant morbidity (Betz 2002, Hill 2010).

A further limitation of the autograft is its availability. As the graft is harvested from the same patient, there is a limited supply and therefore autografts may not be suitable where very large defects arise. The patient's comorbidity status including their smoking habits, may also make the harvesting and integration of the graft riskier, limiting its usefulness and maximising the risk of complications (Betz 2002, Hill 2010).

2.2.2.2. Allograft

In contrast to autografts, allografts are harvested from a separate individual who may be both living or deceased, and are stored in bone banks (Baldwin, Li et al. 2019, Hill 2010). In comparison to autografts, allografts increase the availability of bone graft, including the size of the graft itself, but are only available in countries with the necessary technology. Many regions still do not have access to this capability, due to their lack of appropriate bone banks (Ali, Mubarak et al. 2022, de Alencar and Vieira 2010, Delloye, Cornu et al. 2007). Despite this, they are advantageous in that they do not necessitate the creation of a separate incision in the recipient, eliminating the risk for morbidity associated with what is effectively a second operative procedure (Betz 2002, Hill 2010).

Like autografts, allografted bone may or may not be structural, depending on their intended use (Delloye, Cornu et al. 2007). Unfortunately, since allografts are harvested and implanted within separate individuals, they introduce the possibilities of rejection and transmission of disease. In order to reduce these risks, allografts must be processed in ways which may affect their viability and effectiveness once implanted (Betz 2002, Delloye, Cornu et al. 2007, Fishman, Greenwald et al. 2012, Hill 2010, Zamborsky, Svec et al. 2016).

2.2.2.3. Incorporation of a Bone Graft

Once implanted, a graft must incorporate into the bone within which it is implanted. This takes place through mechanisms which are dependent upon factors such as graft viability and vascularity, and also differ depending on whether the graft is cortical or cancellous in nature (Khan, Cammisa Jr et al. 2005, Stevenson, Emery et al. 1996).

Cancellous bone grafts are incorporated through a process known as creeping substitution. This is primarily an osteoclast-led process where resorption of the grafted bone begins at the interface between host and graft bone. Osteoblasts follow closely behind and lay new, vascularised bone instead. Cortical bone incorporates in a markedly slower process which may weaken the graft considerably, potentially leading to fracture. Like cancellous grafts, this process is primarily osteoclast-led and occurs in the presence of basic multicellular units. It involves the resorption of bone within the osteons with later formation of new haversian systems by osteoblasts in a manner similar to remodelling. Vascularised bone grafts incorporate in a more rapid process which involves the processes described for fracture repair (Miller and Thompson 2019, Ramachandran 2017).

2.2.3. Properties of Bone Grafts

In order for bone grafts to function correctly and incorporate as part of the host's bone, they must exhibit at least one of the following abilities (Albrektsson and Johansson 2001, Giannoudis, Dinopoulos et al. 2005): (1) Osteogenesis; (2) Osteoconduction; (3) Osteoinduction. In reality, autografts tend to feature all three properties and this is what has kept them at the top as the gold standard bone graft.

2.2.3.1. Osteogenesis

Osteogenesis is an ability exhibited by autografted bone, as this already contains within it the biological factors which are able to directly form new bone. As allografted bone is removed from a donor who is often deceased, and subsequently manipulated in order to reduce disease transmission risk, its osteogenic properties are all but lost. There are some reports however of augmentation of the biological properties of allografted bone through the employment of bone marrow aspirate in conjunction with the allograft, which may restore osteogenic potential (Khan, Cammisa Jr et al. 2005, Kwong and Harris 2008).

2.2.3.2. Osteoconduction

As osteoconduction deals with the ability of a bone graft to allow new bone to grow through it, the porous structure of the graft must be taken into account and is of paramount importance. Referring to the vacant spaces within the graft's structure, higher porosities should allow for more bone to form within. Porosity may be split into microporosity, which considers pore diameters below 50µm and macroporosity, which deals with pore sizes above this mark (Hing, Annaz et al. 2005, Wang, Barbieri et al. 2015).

In order for osteoconduction to take place, a bone graft must have an element of macroporosity. The available literature suggests that while bone ingrowth may take place at pore sizes of 50µm diameter, it is much more rapid when pore sizes increase above this level to the 300-500µm region (Chang, Hong et al. 2000, Tsuruga, Takita et al. 1997). In the era of bone graft substitutes, more modern techniques of graft production such as additive manufacturing, appear to allow for an even faster rates of bone formation with pore sizes well in excess of 500µm (De Wild, Ghayor et al. 2019, Weber 2019).

In addition to the size of the pores, their interconnectivity is of interest. Several reports have proven this using both *in vitro* and *in vivo* methods, showing that bone ingrowth best occurs in structures where the connectivity between the pores is improved. A decrease in pore interconnectivity despite an adequate pore size has been shown to decrease the rate at which bone grows through the graft, limiting the osteoconductive potential of the structure (Chang, Song et al. 2016, Lee, Ahn et al. 2010, Li, Habibovic et al. 2007). Some reports suggest that this may at least in part be due to a poor waste-nutrient exchange in grafts with a lower level of pore interconnectivity (Van Bael, Chai et al. 2012).

Undoubtedly, the porous structure of a bone graft has a great effect on the rate at which bone is allowed to grow through its structure. An increased rate of bone formation is desirable within an *in vivo*, clinical situation. In addition to this, is the effect that porosity has on the type of bone that is formed in response. Some studies have shown that particular pore sizes, specifically those in the 100-200µm range, may allow for the primary formation of bone with a mature, haversian structure, including the presence of central blood vessels (Chang, Hong et al. 2000, Kuboki, Takita et al. 1998, Weber 2019).

2.2.3.3. Osteoinduction

This is an important and desirable characteristic of a bone graft. Autografted bone is in a good position to be able to perform this well as it is transported with its MSC and matrix proteins intact. These transported factors are then able to trigger the differentiation of MSC into bone-forming cells, allowing the graft itself to influence and begin the process of bone formation (García-Gareta, Coathup et al. 2015, Goldberg and Akhavan 2005, Khan, Cammisa Jr et al. 2005, Stevenson 1999).

The induction of differentiation of MSC into bone-forming cells may be caused by a myriad of factors. Perhaps the best known and studied of these is the BMP group of proteins. Their effects here are especially pronounced when certain combinations of BMPs are involved. In addition to their bone-differentiating role, BMPs are known to aid in the differentiation of other tissues such as cartilage, where they play an increasingly evident role in endochondral bone healing. Their roles in other sites of the body are also becoming clearer (Katagiri and Watabe 2016, Scarfi 2016).

While BMPs are known to be important in their own right, they are now often implicated in the function of other grafts. One such example is the function of BMPs in conjunction with demineralised bone matrix (DBM). As its name suggests, DBM involves the demineralisation of bone matrix, leaving its biological component (including BMPs) intact. The bone-forming activity of DBM was initially discovered by Urist in 1965 (Urist 1965) but it was not until several years later that this activity was attributed to BMPs. Since that time, this group of proteins has been found to act through multiple pathways, including the Sost/Wnt pathways described earlier. In turn, this leads to differentiation and activation of osteoblasts (Katagiri and Watabe 2016, Rosen 2006). As a result of its appreciable osteoinductive properties, allogeneic DBM has become a mainstay in bone grafting with several commercially available products currently available for use in the clinical setting (Kirk, Ritter et al. 2013, Moore, Katz et al. 2011).

Material composition of the graft has also been shown to have an effect on its osteoinductive capability. Specifically, Ca and related phosphate ceramics such as β -tricalcium phosphate (β -TCP) have been shown to have osteoinductive capabilities, even though they initially received praise for their commendable osteoconductive potential (LeGeros 2008, Tang, Li et al. 2018, Yuan, Yang et al. 1998). Not all calcium phosphate-based ($\text{Ca}_3(\text{PO}_4)_2$)

materials seem to have been created equally however, with β -TCP for example performing better in this regard than HA (Habibovic, Yuan et al. 2005).

In addition to biological signalling and material composition, the graft's structure also appears to have an important effect on its final osteoinductive capabilities. Evidence suggests that the porosity of the graft, in the forms of both microporosity and macroporosity are essential (Chan, Coathup et al. 2012, Habibovic and de Groot 2007, Habibovic, Yuan et al. 2005, Miron and Zhang 2012, Shi, Fang et al. 2022, Zhang, Kurita et al. 2005). Some reports also suggest that materials such as Ti, which are not traditionally considered to be osteoinductive, may be rendered so by altering the graft's structural characteristics (Fujibayashi, Neo et al. 2004).

2.2.4. Bone Graft Substitutes

While autografted bone is considered to be the current gold standard in bone grafting techniques, the harvesting and placement of this form of graft brings with it risks and potential issues that may not be acceptable to every patient. Its alternative, the allograft, while promising to solve some of these issues, also carries significant risk and undeniably does not have the same biological characteristics as autografted bone, meaning that a compromise must therefore be accepted.

As an answer to the issues related to both autografts and allografts, science has turned to other alternatives, these being termed bone graft substitutes. As their name suggests, these are meant to afford a suitable alternative to both autografted and allografted bone. They are intended to remove the potentially detrimental effects related to both of these grafts and bring with them a number of advantages. As bone need not be harvested from a separate site in the same person, potential issues relating to a second surgical site are negated. Additionally,

bone graft substitutes do not require any form of bone bank for storage, decreasing costs and increasing flexibility and availability to the operating surgeon and patient (Finkemeier 2002, Laurencin, Khan et al. 2006, Moore, Graves et al. 2001).

Bone graft substitutes may also be used as structural grafts, depending on their material composition and structure. This method of filling bone defects is frequently employed in injuries such as tibial plateau fractures, these being notoriously associated with bone defects in their subchondral region (Fillingham and Jacobs 2016, Goff, Kanakaris et al. 2013).

2.2.4.1. Ideal Characteristics of a Bone Graft Substitute

It must be emphasised that a single bone graft substitute is unable to cater to all requirements. What may be necessary in some scenarios, such as a rapid corrosion rate and mechanical strength, may not be so in others. Under particular circumstances, such as when a patient's health status is compromised secondary to trauma or other pathology, these may instead become a hindrance. Instead, the operating surgeon must make a judgement call based on the individual patient's needs in order to ensure that the most favourable outcomes are achieved in each case (Janicki and Schmidmaier 2011).

In order for bone graft substitutes to function correctly and replace the function of autografted and allografted bone, they must possess important characteristics. As with autografts and allografts, osteoconduction, osteoinduction and osteogenesis are of utmost importance. Without at least one of these abilities, a bone graft substitute is destined for failure (Faour, Dimitriou et al. 2011, Fernandez de Grado, Keller et al. 2018, Fillingham and Jacobs 2016, Janicki and Schmidmaier 2011).

It would be ideal for bone graft substitutes to additionally possess other characteristics. These should render them capable of performing adequately *in vivo* in the dynamic setting of

bone loss, including in the trauma arena (Faour, Dimitriou et al. 2011, Fernandez de Grado, Keller et al. 2018).

Biocompatibility – Any material or structure to be implanted *in vivo* must be biocompatible if it is to be effective. In the ideal situation, this would mean the complete absence of cytotoxicity, allowing the graft to incorporate into the host's bone as intended. A graft that is toxic will inevitably lead to failure of proliferation of host tissues and bone-forming cells, precluding healing and filling of bone defects, ergo the failure of the bone graft substitute. On the other hand, the materials themselves may have additional beneficial effects, such as Ag's propensity to eradicate bacterial organisms (Hardes, von Eiff et al. 2010, Jung, Koo et al. 2008, Sotoudehbagha, Sheibani et al. 2018).

Degradability – Degradability and biocompatibility go hand in hand. As an implanted material degrades in an *in vivo* environment, it releases degradation products into its surroundings, creating the potential for toxicity. This is especially true for larger implants made of materials known to be poorly biocompatible, or which are frankly cytotoxic. On the other hand, the ability of a structure to degrade once implanted may allow for bone to form within the implant itself, eventually replacing it in its entirety. This may have advantages on many fronts, including the lack of a foreign material to eventually become colonised by infective agents, as may occur for example in haematogenously-transmitted major joint arthroplasty infections (Rakow, Perka et al. 2019).

Structural integrity – As is often the case in critically-sized bone defects, the patient's bone has lost a significant amount of structural support. Operative ORIF methods used in these situations must create an environment where strain is controlled. The addition of a bone graft substitute should ideally be able to lend to the stability of the construct by complementing the fixation method utilised and restoring continuity. As would be the case in tibial plateau

fractures for example, any graft placed beneath the articular surface should lend to the structural integrity, with the ability to hold up the articular surface it is intended to support (Veitch, Stroud et al. 2010).

Safe and easy to use – Any implanted material should be safe. This takes features such as cytotoxicity and biocompatibility into account, but also the risk of transmission of disease. It is known that certain implants used in the past, carry with them the risk of non-communicable diseases such as pseudotumor in the case of metal-on-metal hip resurfacing implants (McConnell, Stambough et al. 2021). Transmissible disease must also be taken into account, as is known to occur in allografted bone. Ease of use lends to the safety of an implant and allows the surgeon to adapt the implant to the patient's specific needs.

2.2.4.2. Available Bone Graft Substitutes

A market search for bone graft substitutes will show that there is an increasing availability of materials. These differ most markedly in composition, being broadly classified into biological and synthetic bone graft substitutes (Campana, Milano et al. 2014, Fernandez de Grado, Keller et al. 2018, Janicki and Schmidmaier 2011, Sohn and Oh 2019).

Perhaps the most prolific of the bone graft substitutes are the ceramics. These are synthetic materials which are most typically Ca-based and often contain HA and β -TCP. These materials are known to be biocompatible and generally possess sought after attributes such as structural integrity, lending to their uses, which may involve the mechanical support of a fracture (Saikia, Bhattacharya et al. 2008, Whitehouse and Blom 2009). Even though these materials may provide some mechanical integrity, their compressive strength is usually more comparable to that of cancellous bone and not of cortical bone (Ryan and Yin 2022). This limits their usability in weight bearing orthopaedic applications, especially of the lower limb. It also

means that patients will likely not be able to bear weight as early as desirable. There are numerous other materials that are currently used to manufacture synthetic bone graft substitutes, including calcium phosphate cements, bioactive glass and polymers, amongst others (Fillingham and Jacobs 2016, Janicki and Schmidmaier 2011, Sohn and Oh 2019).

Biological materials are the flip side of the coin. In this category are included such things as DBM, corals and collagen. Harvested from biological sources, these bone graft substitutes are capable of manipulating the local biological environment at the graft insertion site. This allows for many of the grafts to exhibit osteoinduction in addition to osteoconduction. The majority of these however, show little structural integrity and therefore the operating surgeon must rely on other sources to create a mechanically stable construct for fracture fixation (Campana, Milano et al. 2014, Fillingham and Jacobs 2016, Sohn and Oh 2019). Emphasis is now shifting to a combination of synthetic and biological materials in a single product, benefitting from the structural integrity of ceramics such as HA and also from the biological capabilities of materials such as collagen (Scabbia and Trombelli 2004).

2.2.5. Metal Candidates for Corroding Bone Graft Substitutes

The use of metals in elective orthopaedic practice and fracture fixation is an old and well-tested concept. Many metals have been, and are still being used to this day, the most abundant of which are medical grade stainless steel 316L (SS316L), Ti and cobalt-chrome (Co-Cr). Although these materials are known to undergo wear if used in articulating surfaces such as hip arthroplasty prostheses, they are biocompatible with relatively little effect on surrounding and systemic tissues when this occurs. Their degradation diminishes as these implants are used in non-articulating surfaces such as may occur in ORIF of fractures (Sansone, Pagani et al. 2013).

2.2.5.1. The Dilemma of Bioresorbable Metal Bone Graft Substitutes

In view of their mechanical properties, metals have become candidate materials for the development of bone graft substitutes. However, there lies a problem within this proposition. Commonly used metals such as SS316L and Ti do not corrode to any significant degree. This is by design, corrosion resistance being a feature of these implants which allows them to last for many years *in vivo* and in most cases, outlive the patient (Eliaz 2019, Walczak, Shahgaldi et al. 1998).

The lack of significant degradation over time presents issues with fracture management, especially in young, growing children but also in the presence of complications such as infection. Growing children often require the removal of metallic implants in order to ensure that complications such as the disturbance of growth do not occur. In addition, if infection of the fracture site and implant occur, it is often impossible to eradicate the infection without the removal of the implant itself. This is because a protective biofilm quickly forms on the surface of the implant, shielding bacteria from antibiotic therapy and preventing eradication of infection (Peterson 2005, Rozis, Evangelopoulos et al. 2021, Zoubos, Galanakis et al. 2012). Removing the implant, whether incorporated within the host bone or not, will therefore entail leaving the patient with a critically-sized defect and without the structural support the graft offers. Degradability is therefore an important factor to consider when choosing potential metal candidates for bone graft substitutes.

Balanced against degradation of the implant, is the structural support that the implant can provide, and its biocompatibility. *In Vivo* degradation of the implant releases corrosion products into the surrounding area. These products may accumulate locally, but may also be carried to distant sites such as the liver and kidneys where they may either accumulate or be excreted. In the ideal situation, both the implant itself and its corrosion products are

biocompatible, meaning that they can be handled *in vivo* with little to no risk of toxicity. These products should also not accumulate at distant sites such as the liver, but should instead be eliminated from the body without any harmful effect (Shayesteh Moghaddam, Taheri Andani et al. 2016, Zhang, Jiang et al. 2021).

Structural integrity is another important consideration. Degradation of the implant will inevitably lead to a decrease in the structural support it is able to provide. This may theoretically become an issue in a rapidly degrading implant which does not leave enough time for host bone to grow through it. In these cases, the implant may degrade entirely, leaving unfilled bone void with no structural integrity. The result is a bone defect which will ultimately require further intervention to correct. The flip side of this issue is an implant which is too stiff. Stress-shielding is a well-known issue affecting many non-corroding fracture fixation and arthroplasty implants. This occurs as a consequence of the implants themselves having a structural rigidity and material elastic modulus well above that of the surrounding bone. This may cause resorption of bone (Wolff's law) and a reduction in its strength, predisposing to peri-prosthetic fracture, which is often a very challenging problem (Mi, Shuib et al. 2007, Nagels, Stokdijk et al. 2003, Shayesteh Moghaddam, Taheri Andani et al. 2016).

It is clear that if metal bone graft substitutes are to provide a viable alternative to the traditional autograft and allograft, corrosion rate and cytotoxicity must be balanced. The ideal graft would also provide other benefits such as mechanical integrity, allowing patients to begin weight bearing in the early post-operative period.

2.2.5.2. Magnesium-Based Corroding Bone Graft Substitutes

Mg and its alloys were the first to be studied in depth with regards to a corroding, metallic *in vivo* implant. Reason being that Mg is a common and important cation in human physiology

which the body is able to metabolise and manage efficiently (Wolf and Cittadini 2003). The *in vitro* and *in vivo* study of its structural properties and corrosion resistance have yielded promising results in certain areas such as coronary artery stenting. Here, the implant has been shown to corrode rapidly while providing enough structural support to keep the lumen of the vessel open, at least initially (Chen, Chen et al. 2019, Di Mario, Griffiths et al. 2004).

While Mg has been found to have desirable properties in the realm of vascular stenting, it fails at important stumbling blocks when taking bone grafting into account. The first issue is that it exhibits a corrosion rate which is too rapid to allow for meaningful healing of a critically-sized bone defect to occur. This would ultimately mean that the bone defect initially filled by bone graft substitute would remain a defect following the disappearance of the graft. Although Mg and its alloys show good biocompatibility *in vivo*, they are as yet not adequate for use as bone graft substitutes (Heiden, Walker et al. 2015, Liu, Ren et al. 2018, Shaw, Sikora et al. 2008, Witte, Ulrich et al. 2007a, Witte, Ulrich et al. 2007b).

Despite some evidence suggesting the biocompatibility of Mg-based implants, there also remains an issue with the evolution of H₂ gas. This gas is given off as the implant corrodes and can sometimes be seen on imaging. Some evidence has shown this to interfere with wound healing through cavitation and potentially even tissue damage and necrosis (Celarek, Kraus et al. 2012, Kirkland 2012, Zeng, Dietzel et al. 2008).

Another issue stems from the mechanical properties of grafts fashioned from Mg and relate also to its corrosion rate *in vivo*. At first glance, Mg may seem an appropriate material as the mechanical properties of adequately porous scaffolds are similar to those exhibited by cancellous bone (Wen, Yamada et al. 2004). This would mean that stress shielding problems associated with stiffer materials such as SS316L and Cobalt-Chrome (Co-Cr) may not be an issue with a Mg graft. In spite of this however, this level of rigidity is unlikely to support larger

loads, as would be expected with weight bearing in a lower limb or in the spine. This issue is magnified by the loss of strength which accompanies this materials rapid *in vivo* corrosion (Kirkland 2012).

Because of the issues encountered with Mg, science has turned to other materials such as Zinc (Zn) and Iron (Fe) in order to try and solve the issues encountered (Heiden, Walker et al. 2015, Wang and Yeung 2017).

2.3. Iron-Based Corroding Bone Graft Substitutes

A number of stumbling blocks with Mg and its alloy counterparts have led to interest in Fe as a potential material out of which bone graft substitutes can be manufactured. The reasons for this are numerous but include Fe's inherent and favourable mechanical characteristics, its tendency to corrode, its availability and finally its abundance and importance in the human body's natural physiology.

2.3.1. Mechanical and Corrosion Properties

At atmospheric pressure, pure Fe is polymorphic and may therefore exist as either of two crystalline structures, depending on the temperature to which it is heated. At room temperature, the body-centred cubic (BCC) structure of the alpha allotrope of Fe (α -Fe), called ferrite is stable and exists up to a temperature of 1184K. At this point, a phase transition occurs to γ -Fe, called austenite. Austenite has a different crystalline structure to ferrite, being face-centred cubic (FCC). Once heated to above 1665K the structure reverts once again to a BCC crystalline structure, until the melting point is reached. An important difference between α -Fe and γ -Fe is that the crystalline structure of these allotropes renders α -Fe magnetic and

γ -Fe antiferromagnetic¹. This would mean that implants with γ -Fe should be safe to monitor *in vivo* using modern medical imaging techniques such as MRI, which relies on magnetism for delineation of anatomy and pathology (Pepperhoff and Acet 2001, Singh 2016).

Considering the advantage of having an antiferromagnetic material as an implant, the question rephrases to ask whether or not the austenitic phase γ -Fe, can be stabilised at room temperature. One important method of achieving this is through the alloying of Fe with other elements such as Mn. Increasing the content of Mn within the final alloy increases the stability of γ -FeMn at lower temperatures. With a high enough Mn content therefore, the austenitic phase of FeMn can become stable at room temperature (Raabe, Sun et al. 2020), in addition to maintaining its antiferromagnetic properties. This material and its descendants have since become an important topic of discussion in the field of corroding metal bone graft substitutes (Hermawan, Alamdari et al. 2008, Hermawan, Dubé et al. 2006, Pepperhoff and Acet 2001, Schinhammer, Hänni et al. 2010, Singh 2016, Tonna, Wang et al. 2022, Wang, Tonna et al. 2022).

The austenitic phase of FeMn has been shown to have good ductility and strength (Venezuela and Dargusch 2020), which allows for its improved formability into shapes and forms which may be required for the development of bone graft substitutes. In addition to this, its stiffness is more comparable to that of cortical bone than other commonly used orthopaedic materials such as SS316L. This means that while it should be able to perform well in weight bearing orthopaedic applications, its stress shielding effects are less profound (Čapek, Kubásek et al. 2016, Dargusch, Dehghan-Manshadi et al. 2019).

¹ Antiferromagnetism is a phenomenon seen to occur in certain materials where the anti-parallel arrangement of atomic magnetic moments results in a net magnetic moment of zero.

The final important aspect to consider regarding the ability of a corroding bone graft substitute to perform its functions adequately, is its ability to corrode in an *in vivo* environment. Studies have shown that while FeMn alloys have improved *in vitro* corrosion characteristics when compared to pure Fe, *in vivo* corrosion is still too slow for this alloy to function adequately. This is most likely to occur secondary to the formation of passivation layers on the outer surface of the corroding implant, preventing further attack of the uncorroded material beneath (Čapek, Kubásek et al. 2016, Drynda, Hassel et al. 2015, Venezuela and Dargusch 2020).

A remedy to this problem was suggested through the addition of a noble alloying element to FeMn forming FeMn-X, X denoting the noble element. This was initially suggested to be Palladium (Pd). When incorporated with FeMn in small amounts to form FeMnPd, Pd would allow for the creation of micro-galvanic currents with the effect of accelerating the corrosion rate (Schinhammer, Hänzi et al. 2010). Despite its promise however, this new breed of alloys still exhibited *in vivo* corrosion rates which were too slow (Kraus, Moszner et al. 2014).

Ag has since been proposed as a replacement for Pd in this regard. Evidence has suggested that the addition of small amounts of Ag to FeMn maintains the favourable mechanical characteristics of FeMn. In addition, the creation of micro-galvanic currents may increase the corrosion rate of the resulting FeMnAg alloy (Caligari Conti, Mallia et al. 2019, Sotoudeh Bagha, Khakbiz et al. 2018, Tonna, Wang et al. 2022, Wang, Tonna et al. 2022).

2.3.2. Iron in Human Biology and Pathology

Fe is essential to human physiology. It is an abundant mineral found in several critical processes, from Oxygen (O₂) transport in blood and muscle to the ability of the body to mount an adequate immune response to infection (Beard 2001). The majority of Fe in the body is

found within haemoglobin, the abundant protein molecule within blood which binds O₂ for transport around the body. This portion of Fe is in the ferrous (Fe²⁺) state and constitutes approximately two-thirds of the total amount of Fe found in the body (Beard 2001, Gupta 2014, Winter, Bazydlo et al. 2014).

An average adult human male has within him approximately 4g of Fe, of which around 2.5g is found within erythrocytes and a further 1g can be found within the spleen and liver. The remaining 0.5g is distributed throughout the remainder of the body (Waldvogel-Abramowski, Waeber et al. 2014, Winter, Bazydlo et al. 2014). Fe requirements in the growing child are high but these requirements decrease as adulthood is reached with the vast majority of Fe in the adult being recycled from previous sources such as erythrocytes, and subsequently re-utilised.

Every day, approximately 1-2mg of Fe are lost, mainly through the desquamation of epithelial cells within the gastrointestinal tract. In order to maintain homeostasis, this same amount must therefore be replaced through absorption by enterocytes within the small intestine (Waldvogel-Abramowski, Waeber et al. 2014, Winter, Bazydlo et al. 2014). Ingested Fe is absorbed most avidly in the duodenum and proximal part of the jejunum. The acidic environment within the stomach, in addition to reductase enzymes present at the brush border, aid to stabilise Fe in its ferrous form. Ferrous Fe is then absorbed by enterocytes, mostly through the function of the divalent metal transporter-1 (DMT1) protein. In addition, there are also absorption pathways for both ferric (Fe³⁺) and haem-bound Fe (Fleming and Ponka 2012, Waldvogel-Abramowski, Waeber et al. 2014).

Once absorbed into the enterocyte, Fe is either stored as ferritin or may alternatively be exported by ferroportin, present on the cell membrane of these cells. It may then be transported by transferrin to other areas such as the liver, which is an important store of Fe in the form of ferritin. It is also taken to areas such as bone marrow where Fe then plays an

important role in the production of haemoglobin. Hepcidin is a key controller of Fe absorption and functions to block the ferroportin-mediated exportation of Fe from enterocytes. These cells eventually slough off with their Fe stores within them (Anderson and Wang 2012, Fleming and Ponka 2012, Waldvogel-Abramowski, Waeber et al. 2014, Winter, Bazydlo et al. 2014).

2.3.2.1. Iron Deficiency

Despite the tight regulation of Fe levels within the body, these systems may be overrun in cases where Fe loss is high. Fe deficiency is a prevalent pathology and is commonly seen in women who experience heavy menstrual bleeding. It may also occur secondary to a number of disorders such as inflammatory bowel disease, which interferes with Fe absorption. Deficiency of this essential mineral may result in a number of manifestations, ranging from angular cheilitis and dry mouth to Fe deficiency anaemia (Camaschella 2019, Pasricha, Tye-Din et al. 2021).

2.3.2.2. Iron Overload

The more pertinent issue relating to corroding Fe-based bone graft substitutes is not Fe deficiency but Fe overload. This stems from the expectation that Fe levels within the body will rise as an Fe-based graft corrodes, releasing corrosion products into its surrounding environment.

This condition may be caused by a number of diseases, including disorders of erythroid maturation such as Thalassaemia. This is compounded by the multiple blood transfusions often required in patients who suffer from this disorder. Other diseases such as myelodysplastic disorders are also well known to cause Fe overload. Similarly, abnormalities

of the hepcidin-ferroportin axis, including the well-known haemochromatoses may also bring about this pathology (Andrews 2000, Fleming and Ponka 2012).

Fe overload occurs in two major patterns. The first occurs in conjunction with disorders causing increased catabolism and turnover of red blood cells (erythrocytes). Erythrocytes are broken down in the reticuloendothelial system, of which the spleen and macrophages form an integral part. Macrophages accumulate Fe first and allow this system to absorb a large amount of Fe before it begins to deposit and accumulate in other organ systems. Other disorders will cause plasma Fe levels to rise. In these cases, Fe accumulates very quickly within the liver and spills over to involve other organ systems such as the heart, skin, joints and endocrine organs, very notably within the pancreas (Andrews 2000, Fleming and Ponka 2012, Siah, Trinder et al. 2005). Cellular damage ensues through the reaction of Fe with free radicals, which in turn generates more free radicals (Eid, Arab et al. 2017).

Importantly, abnormally elevated levels of Fe within these organ systems can be detected using non-invasive imaging techniques, most notably MRI. This imaging modality can and is used to both diagnose and monitor levels of Fe in syndromes of Fe overload (Deugnier and Turlin 2007, Palmer, Vishnu et al. 2018, Siddique and Kowdley 2012).

Symptomatology of Fe overload is related to the organ systems affected by the disorder, but can additionally include constitutional symptoms such as weakness and fatigue. One of the first systems to show signs of Fe overload is the skin, hyperpigmentation being a relatively common associated symptom. Deposition of Fe tends to occur most obviously around the sweat glands in sun-exposed regions with the skin taking on a bronze hue as a result. In addition, other signs and symptoms such as hair loss may also occur (Palmer, Vishnu et al. 2018, Siddique and Kowdley 2012). Arthropathy is another of the effects of iron overload, taking on a mostly non-inflammatory arthritic picture characterised by pain and stiffness.

There may also be acute inflammatory episodes resembling calcium pyrophosphate disease and septic episodes, most notably by *Yersinia* species (Faraawi, Harth et al. 1993, Siddique and Kowdley 2012).

Diabetes may occur due to the deposition of Fe within the pancreas. Given the name bronze diabetes in reference to the associated discolouration of the skin, this disease may also be associated with disorders such as hypogonadism. Cardiac arrhythmias may occur in association with cardiomyopathy and heart failure (Palmer, Vishnu et al. 2018, Siddique and Kowdley 2012). Hepatic involvement may be marked and is due to fibrosis and eventual cirrhosis, causing liver failure. In its worst form, this cascade of events may even lead to malignancy, especially hepatocellular carcinoma (Deugnier and Turlin 2007, Palmer, Vishnu et al. 2018, Siddique and Kowdley 2012).

2.3.3. The Biology of Manganese

As important as Fe is for normal human physiology, other elements such as Mn also play essential roles. Mn is a nutrient obtained from water and food sources and similar to Fe, it is absorbed by the gastrointestinal tract. Once absorbed it is transported to, and concentrated within specific organs such as the liver, brain and endocrine organs, especially the pancreas (Chen, Bornhorst et al. 2018, Li and Yang 2018).

Mn appears to have important roles in several areas, one of the best known being the synthesis and function of specific enzymes such as lyases, transferases and oxidoreductases, among others. Mn superoxide dismutase is a particularly important enzyme which requires Mn in order for it to function correctly. Its role is to scavenge the reactive oxygen species (ROS) produced during mitochondrial activity, explaining why Mn is concentrated in mitochondrial

rich cells (Chen, Bornhorst et al. 2018, Holley, Bakthavatchalu et al. 2011, Salomon, Keren et al. 2011).

As Mn is a trace element found in several dietary sources, it is only required in small amounts. Deficiency is therefore rare but may result in several profound medical issues ranging from issues with bone formation and growth to altered glucose tolerance and diabetes (Horning, Caito et al. 2015, Li and Yang 2018, Soares, Silva et al. 2020).

Mn toxicity is a more commonly encountered medical issue and is of particular interest as a potential complication of corroding FeMn bone graft substitutes. Toxicity of Mn is known to cause neurological abnormalities, specifically manganism. This is a disorder which is seen to closely resemble Parkinson's disease, wherein the patient experiences symptoms such as tremors and difficulties with gait which are unresponsive to conventional dopamine-based Parkinson's disease therapies (Aschner, Guilarte et al. 2007, Balachandran, Mukhopadhyay et al. 2020, O'Neal and Zheng 2015, Santamaria 2008, Soares, Silva et al. 2020).

2.3.4. The Biology of Silver

Ag is a rather rare element but nonetheless, it has been used throughout history. One of the earliest accounts of its use include the carriage of water, especially on the battlefield, as it kept water fresh and potable for extended periods of time (Alexander 2009). Since then, it has been used medically on various occasions and as part of several medical treatments, from prostheses to dressings (Drake and Hazelwood 2005).

Despite its varied use in health-related aspects, Ag is not currently known to have any essential biological function in humans and for this reason, deficiency of Ag is not known to have any ill-effect. Consequently, its uses in modern medicine are generally limited to its known anti-microbial actions (Alexander 2009, Betts, Whitehead et al. 2020). It is understood

that Ag has anti-microbial functions which it exerts through a multi-modal fashion. There are a number of mechanisms which are employed, including the disruption of bacterial cell membranes and cell walls, interference with bacterial DNA and the generation of ROS which damage the bacterial cell, among others (Betts, Whitehead et al. 2020).

Its use in this regard is not completely risk-free however. Despite its very rare occurrence, Argyria may be the effect of chronic exposure to elevated levels of Ag. Being derived from the Greek word for silver, Argyria describes a dermatological condition characterised by the bluish-grey discolouration of the skin. This is due to the deposition of insoluble silver compounds in the subdermal layer. While Ag's effect on skin is not thought to be dangerous in itself, some reports suggest other ill-effects such as the derangement of liver function and disturbance of gastro-intestinal flora. Yet other *in vitro* reports show that Ag may be toxic to mammalian cells (Ahamed, AlSalhi et al. 2010, Baker, Leidy et al. 2011, Betts, Whitehead et al. 2020, Das, Petrof et al. 2015, Drake and Hazelwood 2005, Jerger and Parekh 2021, Trop, Novak et al. 2006).

2.3.5. Cytotoxicity and Biocompatibility

2.3.5.1. Pure Iron

The implantation of any foreign material into the body comes with potential associated risks of cytotoxicity and ill-effect. Certain Fe-based materials such as SS316L are resistant to corrosion in view of the presence of a passive film on their surfaces and have been proven to be biocompatible time and time again. The game changes when considering corroding Fe-based bone graft substitutes however (Noor, Jamaludin et al. 2017, Noviana, Estuningsih et al. 2015), where the focus of study becomes the balancing of two integral elements of such an implant; cytotoxicity and the intimately linked corrosion. While essential to the proper

function of a degradable implant, an increasing corrosion rate may predispose to the release of toxic materials at a rate with which human physiology is not able to cope. On the other hand, a corrosion rate which is too slow will not allow the graft to experience appreciable corrosion and therefore the graft cannot be classed as a truly corroding implant. Either way, failure on either one of these fronts will essentially mean the failure of the graft. For this reason, a balance must be struck between these two competing elements.

Pure Fe was the first to be studied with regards to its cytotoxicity and corrosion resistance in both the fields of orthopaedics and coronary artery stenting. It showed immediate promise with Zhu, Huang et al. (2009) performing indirect *in vitro* cytotoxicity tests using Human umbilical vein endothelial cells (HUVEC). In their experiment, they noted favourable cytotoxicity for pure Fe, even commenting on a possible increase in these cells' metabolic activity at particular concentrations of Fe. It was only at significantly higher Fe concentrations that any negative effect was noted (Zhu, Huang et al. 2009).

A similar result was obtained by Moravej, Purnama et al. (2010). In their experiment, rat vascular smooth muscle cells were exposed in an indirect manner to pure Fe, with SS316L as a control material. They noted no decrease in the metabolic activities of the cells exposed to Fe, these being comparable to SS316L (Moravej, Purnama et al. 2010).

Despite the promising results with regards to cytotoxicity, both of these experiments exposed a flaw in pure Fe's capabilities as a bioresorbable material. These experiments evidenced very little *in vitro* corrosion for structures formed of pure Fe (Moravej, Purnama et al. 2010, Zhu, Huang et al. 2009).

A slightly different experiment was performed by Nie, Zheng et al. (2010). Both indirect and direct methods were employed in this experiment with indirect tests evidencing acceptable cytotoxicity of pure Fe to both L929 (mouse fibroblast cell line) and ECV304 (human umbilical

vein endothelial cell line). Both of these cell lines were also seen to proliferate on the surfaces of pure Fe samples. In contrast, there was significant cytotoxicity to vascular smooth muscle cells with these cells exhibiting decreased viability over the test period and poor proliferation over the surfaces of pure Fe samples. Despite this, pure Fe was not seen to cause significant haemolysis to human blood samples. In this experiment, as in others, corrosion was noted to be slow and was likely to be secondary to passivation layers forming on the surfaces of the pure Fe samples (Nie, Zheng et al. 2010).

The remaining *in vitro* literature shows that pure Fe may in fact cause cytotoxicity (Robles Cuellar 2021), especially when cells are exposed to higher concentrations of its corrosion products (Zhang, Chen et al. 2010). It also became clearer that differing methods of cytotoxicity testing may yield different results. One particular study by Scarcello, Herpain et al. (2020) showed that human endothelial and smooth muscle cells grown in direct contact with Fe-based materials may suffer increased cytotoxicity as compared to those exposed to extracts. Ultimately, there was further evidence of an element of cytotoxicity caused by Fe (Scarcello, Herpain et al. 2020). This was also seen by Sharma, Jain et al. (2020), who reported that murine fibroblast cells grown in direct contact with varying structures of Fe may also suffer different cytotoxicity effects (Sharma, Jain et al. 2020).

Peuster, Wohlsein et al. (2001) and Peuster, Hesse et al. (2006) performed important *in vivo* work where pure Fe was investigated for its potential as a degrading metal stent in the cardiovascular system. In these works, New Zealand white rabbits (Peuster, Wohlsein et al. 2001) and minipigs (Peuster, Hesse et al. 2006) underwent operative procedures where pure Fe stents were deployed within their descending aortas. Both of these studies yielded similar results in that there were no appreciable cytotoxic effects seen, even after periods of implantation extending up to eighteen months. This was true for both the local cellular

environment in direct contact with the stent and for systemic organs such as the liver, spleen, heart and para-aortic lymph nodes, where Fe is expected to concentrate. In the 2006 study performed by Peuster, Hesse et al. (2006) on minipigs, Fe was found within specific organs including the spleen and lymph nodes but even so, no cytotoxic effects were noted. Despite these positive findings, the stent itself was noted to have undergone very little corrosion, not sufficient for Fe to be classed as a truly corroding material for corroding bone graft substitutes (Peuster, Hesse et al. 2006, Peuster, Wohlsein et al. 2001).

These positive *in vivo* findings with regards to biocompatibility have been mirrored by other groups who report that pure Fe may have a favourable cytotoxicity profile that is comparable to industry standard non-corroding materials such as Co-Cr. Still however, corrosion rate was unacceptably slow (Waksman, Pakala et al. 2008, Wegener, Behnke et al. 2021, Wegener, Sichler et al. 2020, Wu, Hu et al. 2012). Both *in vitro* and *in vivo* evidence regarding pure Fe is summarised below in Table 1.

Table 1 – Table showing the evidence base relating to the *in vitro* cytotoxicity, *in vivo* biocompatibility and corrosion attack of pure Fe.

Author/s	Cytotoxicity/Biocompatibility	Corrosion Attack
<u>In Vitro</u>		
Zhu, Huang et al. (2009)	Good	Slow corrosion
Moravej, Purnama et al. (2010)	Good	Slow corrosion
Nie, Zheng et al. (2010)	L929/ECV304 – Good Smooth muscle cells – Toxic	Slow corrosion
Zhang, Chen et al. (2010)	Toxic at higher Fe concentrations	Slow corrosion
Scarcello, Herpain et al. (2020)	Greater toxicity in direct contact tests	-
Sharma, Jain et al. (2020)	Toxicity with higher Fe concentrations and when grown on different structures of Fe	Slow corrosion
Robles Cuellar (2021)	Toxic	Slow corrosion
<u>In Vivo</u>		
Peuster, Wohlsein et al. (2001)	Good	Slow corrosion
Peuster, Hesse et al. (2006)	Good	Slow corrosion
Waksman, Pakala et al. (2008)	Good	Slow corrosion
Wegener, Sichler et al. (2020)	Good	Slow corrosion
Wegener, Behnke et al. (2021)	Good	Slow corrosion

2.3.5.2. Iron-Manganese

The addition of alloying elements to pure Fe introduced the possibility of increasing the corrosion rate, but at the potential expense of an increased cytotoxicity profile. The addition of Mn has allowed for the formation of an austenitic material with antiferromagnetic properties, creating the possibility for compatibility with MRI.

Initial *in vitro* efforts by Hermawan, Purnama et al. (2010) showed a high toxicity for pure Mn on murine fibroblasts, as compared to controls of SS316L and pure Fe. Fe₂₅Mn and Fe₃₅Mn on the other hand exhibited a cytotoxicity profile which lay somewhere between that of controls and pure Mn. This however came with what appeared to be a favourable, increased corrosion rate (Hermawan, Purnama et al. 2010).

The following years saw a gradual increase in the work performed on FeMn alloys. In view of the apparent toxicity of pure Mn reported by Hermawan, Purnama et al. (2010), other groups have used differing proportions of Mn by weight in the final alloy. Relatively low proportions of Mn were used by Drynda, Hassel et al. (2015), who performed *in vitro* cytotoxicity tests on primary HUVEC and smooth muscle cell lines. In their experiments, FeMn alloys containing between 0.5% and 6.9% Mn by weight were not seen to cause any significant cytotoxicity (Drynda, Hassel et al. 2015).

Considering the favourable results obtained with low proportions of Mn in the final alloy, work began to focus on higher Mn concentrations. Fe₃₀Mn was investigated by Čapek, Kubásek et al. (2016), who performed indirect cytotoxicity tests on an L929 cell line. This group found the cytotoxicity profile for Fe₃₀Mn to be favourable, with metabolic activities of the tested cells decreasing by only minor degrees as compared to pure Fe. Contrary to what was hoped however, the Fe₃₀Mn alloy was actually found to corrode at a slower rate than pure Fe, yielding a result which contrasted with the findings of Hermawan, Purnama et al. (2010)

(Čapek, Kubásek et al. 2016, Hermawan, Purnama et al. 2010). Other work has since also been performed which tends to show these alloys' good biocompatibility, but with the suggestion that increasing proportions of Mn may increase cytotoxicity (Liu, Zhang et al. 2020).

One work in particular has contrasted these results significantly. Robles Cuellar (2021) looked at the cytotoxicity of FeMn on an hFOB cell line and showed a significant cytotoxicity profile in both direct and indirect *in vitro* cytotoxicity tests. Even so, corrosion rate was still seen to be poor overall (Robles Cuellar 2021).

As happened with pure Fe, FeMn has also shown the potential to cause more toxicity in direct contact situations. Caligari Conti, Aquilina et al. (2018) showed that an hFOB cell line exposed to extracts of Fe₁₃Mn-1.2C exhibited a more significant reduction in viability if the extract contained solid corrosion debris. This was as opposed to extracts which had been centrifuged and therefore contained no such insoluble debris (Caligari Conti, Aquilina et al. 2018). Similarly, Putra, Leeflang et al. (2021) found Fe₂₀Mn, Fe₃₀Mn and Fe₃₅Mn to cause significant toxicity to directly exposed MC3T3-E1 cells. When these were contrarily exposed to extracts, toxicity waned as the concentration of corrosion products within the extract decreased (Putra, Leeflang et al. 2021).

Results with regards to corrosion rate have been conflicting. Dargusch, Dehghan-Manshadi et al. (2019) performed *in vitro* tests using FeMn of varying Mn proportions (Fe₂₀Mn, Fe₃₀Mn and Fe₃₅Mn). In their work, this group noted the corrosion rate of Fe₃₅Mn to be greatest, with cytotoxicity on MC3T3-E1 cells also being most favourable with this particular material (Dargusch, Dehghan-Manshadi et al. 2019). A similar Fe₃₅Mn material was also tested by Carluccio, Xu et al. (2019), who also evidenced good MC3T3-E1 cell growth both directly on the materials themselves, but also through indirect testing (Carluccio, Xu et al. 2020).

Shuai, Yang et al. (2020) performed both direct and indirect cytotoxicity tests in their work, with encouraging results. This group evidenced only minimal cytotoxicity of Fe₂₅Mn scaffolds in both tests. Further imaging of the directly seeded samples after a maximum of 5 days showed a high density of MG63 cells (human osteosarcoma cell line) on their surfaces. In contrast to Dargusch, Dehghan-Manshadi et al. (2019) however, this group also evidenced a higher corrosion rate of Fe₂₅Mn than of pure Fe (Dargusch, Dehghan-Manshadi et al. 2019, Shuai, Yang et al. 2020). These results with regards to cytotoxicity and corrosion rate have been mirrored by other groups (Nie, Chen et al. 2021).

Despite some groups evidencing favourable *in vitro* corrosion rates of FeMn materials, it is still not rapid enough for them to function adequately as bioresorbable bone graft substitutes (Čapek, Kubásek et al. 2016, Dargusch, Dehghan-Manshadi et al. 2019, Dehestani, Trumble et al. 2017, Hermawan, Alamdari et al. 2008, Hermawan, Dubé et al. 2010, Shuai, Yang et al. 2020, Tonna, Wang et al. 2022).

In vivo studies are still lacking with very little evidence being available. One study published in 2015 shows good biocompatibility *in vivo*, but this work deals with amounts of Mn in the region of 0.5% – 6.9% by weight of the final FeMn alloy. This likely signifies that the tested alloys were not austenitic as the amount of Mn by weight was significantly lower than required to produce an austenitic FeMn material which is stable at room temperature (Drynda, Hassel et al. 2015).

A more promising result was published by Nie, Chen et al. (2021) in an experiment which took an Fe₃₀Mn alloy into account. *In vivo* performance of this alloy was found to be good after a 48 week implantation period within the femora of rabbits. No local cytotoxicity was noted along with no ill effect at distant sites such as the liver and kidneys. Results also tended to show an increased rate of corrosion and loss of strength of the implant. Still, corrosion at

48 weeks was documented as inadequate for the purposes of a corroding bone graft substitute.

Traverson, Heiden et al. (2018) investigated an alloy of similar composition. In their experiment, wires formed from Fe30Mn were implanted within the femora of rats. Histological examination evidenced areas of what was termed mild local irritation surrounding the implants. Despite this, overall biocompatibility was found to be good (Traverson, Heiden et al. 2018).

A final *in vivo* study was performed by Carluccio, Xu et al. (2019). In their study, Fe35Mn scaffolds were implanted into defects created within the skull bones of Sprague Dawley rats. This group noted bone to be growing in contact with the scaffold after only 4 weeks of implantation, evidencing good biocompatibility (Carluccio, Xu et al. 2020). Both *in vitro* and *in vivo* evidence regarding FeMn is summarised below in Table 2.

Table 2 – Table showing the evidence base relating to the *in vitro* cytotoxicity, *in vivo* biocompatibility and corrosion attack of FeMn alloys.

Author/s	Cytotoxicity/Biocompatibility	Corrosion Attack
<u>In Vitro</u>		
Hermawan, Purnama et al. (2010)	25%, 35% Mn, Increased toxicity with increasing Mn content	Faster corrosion of Fe25Mn than Fe35Mn
Drynda, Hassel et al. (2015)	0.5 – 6.9% Mn, Good	Slower corrosion of FeMn than of pure Fe
Čapek, Kubásek et al. (2016)	Increased toxicity with increasing Mn content	Slower corrosion of FeMn than of pure Fe
Caligari Conti, Aquilina et al. (2018)	12% Mn, Increased toxicity with particulate corrosion	Slow corrosion
Dargusch, Dehghan-Manshadi et al. (2019)	20%, 30%, 35% Mn, Good	Fastest corrosion of Fe35Mn
Carluccio, Xu et al. (2020)	20%, 30%, 35% Mn, Good	Fastest corrosion of Fe35Mn
Shuai, Yang et al. (2020)	25% Mn, Minimal toxic effect	Faster corrosion of Fe25Mn
Putra, Leeflang et al. (2021)	20%, 30%, 35% Mn, Toxic in direct contact and with increasingly concentrated extracts	Slow corrosion of all materials
Nie, Chen et al. (2021)	30% Mn, Good	Slow corrosion
Robles Cuellar (2021)	35% Mn, Toxic	Slow corrosion
<u>In Vivo</u>		
Drynda, Hassel et al. (2015)	0.5 – 6.9% Mn, Good	Slow corrosion of all materials
Traverson, Heiden et al. (2018)	30% Mn, Good	Slow corrosion
Carluccio, Xu et al. (2020)	35% Mn, Good	Slow corrosion
Nie, Chen et al. (2021)	30% Mn, Mild local irritation with areas of bone necrosis	Slow corrosion

2.3.5.3. Iron-Manganese-Silver

The addition of Ag to FeMn alloys brings with it the promise of an increased corrosion rate, a feature which FeMn alloys thus far have appeared to lack. The trade-off however, is the increase potential for cytotoxicity on two fronts. The first is due to the normal human physiology's lack of Ag, meaning that this element may build rapidly and become toxic in small quantities. The second is presented by the increased corrosion rate that this element may bring about in Fe-based alloys.

To date, there is very little evidence to showcase the cytotoxicity, biocompatibility and corrosion resistance of FeMnAg alloys, both *in vitro* and *in vivo*. The first *in vitro* evidence of cytotoxicity relating to Ag-containing Fe-based alloys came from a work performed by Sotoudehbagha, Sheibani et al. (2018) on a HUVEC cell line. In this work, indirect cytotoxicity tests were performed using Fe30Mn, Fe30Mn1Ag and Fe30Mn3Ag alloys. This study's results evidence signs of toxicity relating to all three alloys, but were most marked in the higher Ag-content alloys towards the end of the testing period. This was most likely the result of a higher Ag⁺ concentration and its resultant cytotoxicity, in the extract obtained with Fe30Mn3Ag as opposed to Fe30Mn1Ag. Similarly, antibacterial effect was most marked in the 3% Ag-content alloy, likely due to similar reasons for increased mammalian cell toxicity (Sotoudehbagha, Sheibani et al. 2018).

Slightly different results regarding cytotoxicity were obtained in a similarly designed study performed by Bagha, Khakbiz et al. (2020). In this particular work, Fe30Mn, Fe30Mn1Ag and Fe30Mn3Ag alloys were tested for cytotoxicity using indirect techniques and using a HUVEC cell line. The higher Ag-content Fe alloys showed the most favourable cytotoxicity profile with no decrease in cell viability over the testing period. This was put down to the somewhat

unexpected result obtained where the Fe30Mn non-Ag-containing alloy exhibited the fastest corrosion rate overall (Bagha, Khakbiz et al. 2020).

Indirect cytotoxicity tests were also performed by Liu, He et al. (2019), this time pitting only Ag-containing Fe30Mn alloys against each other. The four alloys tested contained 1%, 2%, 5% and 10% Ag by weight of the final alloy. Dilutions (10%) of the obtained extracts showed signs of cytotoxicity on a HUVEC line. The most favourable cytotoxicity profile was exhibited by the higher, 10% Ag-content alloy with the 1% and 2% Ag alloys falling short of the *ISO 10993-5:2009, Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity* target, set at 70% viability. The 5% Ag alloy also exhibited an acceptable cytotoxicity profile, despite it being the fastest to corrode (Liu, He et al. 2019).

One *in vitro* experiment was performed recently and showcased both direct and indirect cytotoxicity of pure Fe, Fe35Mn, Fe35Mn1Ag and Fe35Mn5Ag on an hFOB cell line. Despite the previously inspiring evidence with regards to Ag-containing FeMn alloys, this particular experiment showed a rather different result. All of the tested materials exhibited very significant cytotoxicity, both in direct and indirect contact tests (Robles Cuellar 2021).

In vivo experimentation is severely lacking. There is seemingly only a single work addressing the biocompatibility of Ag-containing Fe-based alloys *in vivo*. This work, performed by Dargusch, Venezuela et al. (2020), compares the behaviour of Fe35Mn and Fe35Mn1Ag implanted into rats against a reference WE43 (Mg alloy containing Yttrium, Neodymium and Zirconium, Mg-3.5Y-2.3Nd-0.5Zr) material. In contradiction to the previously noted *in vitro* studies, this work evidenced a more rapid corrosion rate of the Ag-containing alloy *in vivo* as compared to Fe35Mn. Biocompatibility was seen to be good for both implants. Although an element of gas formation is expected in relation to the WE43 material, gas was also seen to evolve around both the Fe35Mn and Fe35Mn1Ag implants, albeit to a lesser extent. Slightly

more gas evolution was in fact noted around the Ag-containing implant (Dargusch, Venezuela et al. 2021). Both *in vitro* and *in vivo* evidence regarding FeMnAg is summarised below in Table 3.

Table 3 – Table showing the evidence base relating to the *in vitro* cytotoxicity, *in vivo* biocompatibility and corrosion attack of FeMnAg alloys.

Author/s	Cytotoxicity/Biocompatibility	Corrosion Attack
<u>In Vitro</u>		
Sotoudehbagha, Sheibani et al. (2018)	Fe30Mn and Fe30MnAg with 1%, 3% Ag, Increasing toxicity with increasing Ag	Slower corrosion of Ag-containing alloys than of non-Ag-containing alloys
Liu, He et al. (2019)	Fe30MnAg with 1%, 2%, 5% and 10% Ag, Least toxicity with 10% Ag	Fastest corrosion with 5% Ag-containing alloy
Bagha, Khakbiz et al. (2020)	Fe30Mn and Fe30MnAg with 1%, 3% Ag, 3% Ag least toxic, Fe30Mn most toxic	Faster corrosion of Fe30Mn than of Ag-containing alloys
Robles Cuellar (2021)	Fe35Mn and Fe35MnAg with 1%, 5% Ag, Toxic	Slow corrosion of all materials
Dargusch, Venezuela et al. (2021)	Fe35Mn and Fe35Mn1Ag, No <i>In vitro</i> cytotoxicity	Fastest corrosion of Ag-containing material
<u>In Vivo</u>		
Dargusch, Venezuela et al. (2021)	Fe35Mn and Fe35Mn1Ag, Good	Fastest corrosion of Ag-containing material

Methodology

3.1. Preparation of Materials

3.1.1. Reagents, Materials and Equipment

A number of reagents, materials and equipment were necessary for the manufacture and preparation of test Fe alloy coupons, SS316LVM coupons and Thermanox™ coverslips. These are listed in Tables 4, 5 and 6.

Table 4 – Reagents used for the preparation of test Fe alloy coupons, SS316LVM coupons and Thermanox™ coverslips – Continued on two pages.

<u>Reagent</u>	<u>Brand</u>	<u>Function</u>
Ag powder	Alfa Aesar, Germany	99.99% purity Ag powder used to manufacture Fe-based test metal coupons
Fe powder	US Research Nanomaterials Inc., USA	99% purity Fe powder used to manufacture pure Fe test metal coupons
Mn powder	Alfa Aesar, Germany	99.6% purity Mn powder used to manufacture Fe-based test metal coupons
SS316LVM	Klein, Switzerland	This stainless steel is used in medical-grade equipment including within implants such as plates and screws for fracture fixation. It is resistant to corrosion and was used in this case as a control material for <i>in vitro</i> cytotoxicity tests

Table 4 – Reagents used for the preparation of test Fe alloy coupons, SS316LVM coupons and Thermanox™ coverslips – Continued on two pages.

Thermanox™	Nunc™, Thermo Fisher Scientific, USA	Formed of a proprietary polyester material and resistant to several chemicals such as ethanol, these coverslips are cell-culture treated and allow for the adherence and growth of cells on their surfaces
Deionised water		Used to dilute solutions such as ethanol to the required strength while ensuring the maintenance of chemical purity
Hexamethylenetetramine (HMTA)	Sigma-Aldrich®, USA	A chemical added to acids such as HCl as part of an acid etching solution. Its adsorption to the surface of Fe alloy coupons inhibits their corrosion while permitting HCl to react with MnO
Hydrochloric acid (HCl)	Sigma-Aldrich®, USA	Using a stock solution of 12M HCl, a 1M solution of HCl was created as part of an acid etching solution. This was used to remove MnO from the test metal coupons before <i>in vitro</i> and <i>in vivo</i> experiments were performed
Isopropyl alcohol (IPA) (99%)	Gibco™, Thermo Fisher Scientific, USA	This concentration of IPA was used for the preparation and cleaning of the test metal coupons. More dilute preparations could not be used as they contain water and would therefore have allowed the coupons to begin rusting before their use in <i>in vitro</i> and <i>in vivo</i> tests
Diamond polishing suspensions (6µm and 3µm)	Struers®, Denmark	Diamond suspensions used with appropriate polishing cloths for the polishing of material surfaces
OP-S polishing suspension (<1µm)	Struers®, Denmark	Silica suspension used for polishing material surfaces. Achieves a very fine finish which is ideal for material characterisation

Table 5 – Materials used for the preparation of test Fe alloy coupons, SS316LVM coupons and Thermanox™ coverslips.

<u>Material</u>	<u>Brand</u>	<u>Function</u>
SiC grinding paper (P400 – P2500)	MetPrep, UK	SiC (Silicon Carbide) grinding paper loaded onto a rotatory grinding machine and used to achieve the desired finish on test coupons
Polishing pads	MD-Dac and MD-Chem, Struers®, Denmark	Polishing cloths used with the appropriate diamond (MD-Dac) and OP-S (MD-Chem) suspensions to achieve finishes of up to 3µm and <1µm respectively

Table 6 – Equipment used for the preparation of test Fe alloy coupons, SS316LVM coupons and Thermanox™ coverslips – Continued on two pages.

<u>Equipment</u>	<u>Brand</u>	<u>Function</u>
Precision scale	Precisa, Switzerland	Precision weighing scale with a resolution of 0.1mg. Used to weigh out individual powders in order to ensure adequate proportions by weight of each coupon. Also used to weigh out 10g portions of mixed powders for the manufacture of Fe alloy coupons
Tumbler	Inversina, Bioengineering AG, Switzerland	Tumbler used to mix batches of powders before their separation into 10g batches for individual coupons
Hydraulic press	Instron 8802, Instron, USA	Hydraulic press which is often used in fracture mechanics and fatigue testing. It may also be used, as in this case, to press powders in powder metallurgy and sintering procedures

Table 6 – Equipment used for the preparation of test Fe alloy coupons, SS316LVM coupons and Thermanox™ coverslips – Continued on two pages.

Furnace	Tube furnace, Nabertherm, Germany	Tube furnace with the capability of reaching temperatures in excess of 1300°C. Allows control of ramp rate and is an essential part of the sintering process. Also allows the introduction of specific gases to achieve a partially reducing atmosphere
Cutting machine	Accutom, Struers®, Denmark	Uses a spinning disc to perform its cutting function. SS316LVM rods were cut into individual coupons using this piece of equipment
Grinding machine	LaboSystem, Struers®, Denmark	Manual grinding equipment used in conjunction with specific grit grinding paper and polishing pads to achieve the desired finish on the surfaces of the Fe alloy and SS316LVM coupons
Gold sputter-coater	Automatic Sputter Coater, Agar, USA	Automated gold sputter-coating system which allows for accurate SEM imaging of coupons and pins in <i>in vitro</i> and <i>in vivo</i> experiments respectively
Scanning Electron Microscope	FE-SEM, Merlin Gemini II column, Carl Zeiss AG, Germany	Scanning electron microscope used for the imaging of coupons and pins. EDS analysis was also made possible using an AMETEK® EDAX detector (USA)

3.1.2. Biodegradable Iron Alloy Coupons

3.1.2.1. Powder Preparation and Sintering

Fe-based metal coupons and pins were produced for the purposes of *in vitro* cytotoxicity and *in vivo* biocompatibility testing respectively. This was performed with the help of Ms. Christabelle Tonna, as part of her ongoing doctoral work. The coupons and pins were manufactured to contain known amounts of specific elements as demonstrated in Table 7. The coupons used for *in vitro* testing with both hFOB and MC3T3-E1 cell lines were produced

concurrently through a process of powder metallurgy. Some of these coupons were then machined into pins. The size and shape of the manufactured coupons is depicted in Figure 2 while that of the pins is depicted in Figure 3.

Table 7 – Powders used to manufacture the test Fe-alloy coupons.

Material	%wt. Fe	%wt. Mn	%wt. Fe:Mn	%wt. Ag
Pure Fe	100	0	-	0
Fe35Mn	65	35	65:35	0
Fe35Mn1Ag	64.35	34.65	65:35	1
Fe35Mn5Ag	61.75	33.25	65:35	5

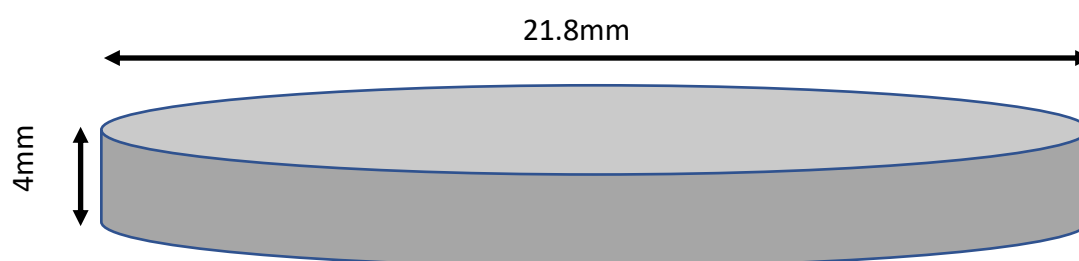


Figure 2 – Dimensions and shape of the coupons used for *in vitro* cytotoxicity testing.

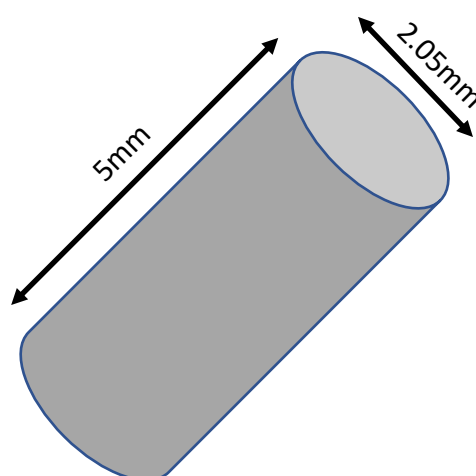


Figure 3 – Fe-alloy pin machined to 2.05mm diameter and 5mm length.

The Fe powder (99% purity, < 45 μ m) used was supplied by US Research Nanomaterials Inc. (USA). The Mn powder (99.6% purity, < 10 μ m) and the Ag powder (99.9% purity, 4-7 μ m) were both supplied by Alfa Aesar (Germany). Powders were weighed using a Precisa precision scale (Switzerland) with a resolution of 0.1mg. The powders were then mixed in the ratios listed in Table 7 in order to produce the austenite phase of FeMn. The resulting powder mixes were then placed in an Inversina tumbler (Bioengineering AG, Switzerland) with five stainless steel balls, each of 10mm diameter. These were added to aid mixing. Mixing took place over a period of six hours in order to ensure that homogenous powder mixtures were obtained. Once thoroughly mixed, the stainless steel balls were removed and the powders were divided into portions of 10g $\pm$ 10mg for individual coupons to be pressed. The resulting 10g portions were individually placed in a die with a diameter of 21.8mm, as shown in Figure 4, and uni-axially pressed at 442MPa using an Instron 8802 hydraulic press (USA). This yielded individual coupons of 10g $\pm$ 10mg weight.

The coupons were then sintered in a tube furnace (Nabertherm, Germany) at 1120°C for three hours, following a ramp rate of 180°C/h. This took place in a partially reducing atmosphere with N₂-5H₂ flowing at a rate of 100 L/h throughout the whole heat treatment, including furnace cooling. This produced metal coupons of similar size and shape to be used for direct cytotoxicity tests.

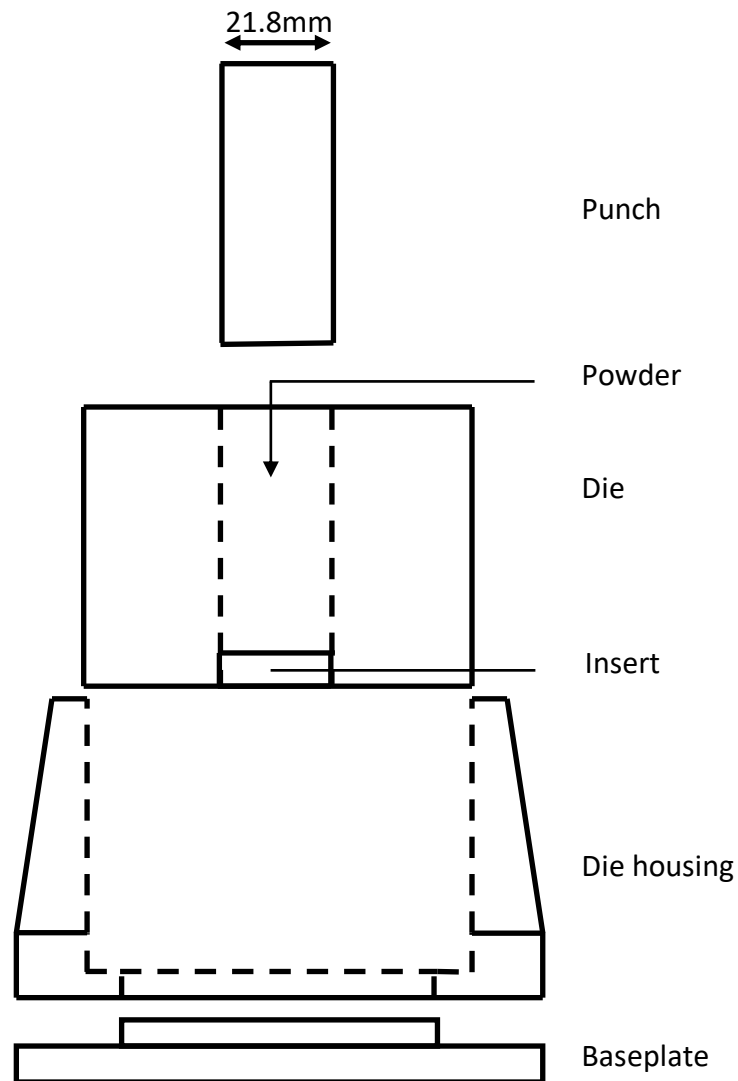


Figure 4 – Diagram of the die used to uni-axially press the test Fe alloy coupons. The diameter of the resultant coupons was 21.8mm.

3.1.3. Preparation of Stainless-Steel Coupons

Stainless steel coupons were cut from a 25mm diameter rod of SS316LVM, acquired from Klein (Switzerland). The diameter was reduced to 21.9mm diameter using a lathe and individual coupons, each of 4.1mm depth were cut using a Struers® Accutom (Denmark) cutting machine. These were then ground in the same manner as the Fe-alloy coupons to a

final grit of P2500, yielding Fe-alloy and SS316LVM coupons of the same dimensions, as shown in Figure 2. This ensured the same surface area for attachment of cells on all coupons.

3.1.4. Preparation of Thermanox™ Coverslips

Thermanox™ coverslips were obtained from Nunc™, (Thermo Fisher Scientific, USA) in a rectangular size of 24mm x 30mm. These were then cut to size with the help of a laser cutting tool and the excess edge bent to create a lip where the coverslip could be handled, as shown in Figure 5.

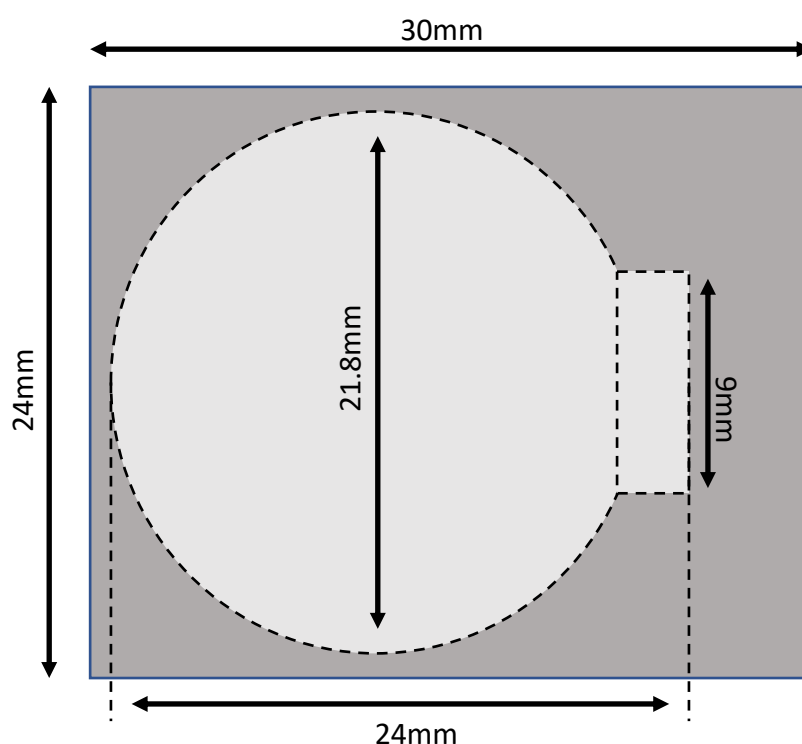


Figure 5 – Thermanox™ discs with a grab area cut from rectangular sheets of 30mm length, 24mm width and 0.2mm thickness.

3.1.4.1. Surface Preparation

3.1.4.1.1. Grinding

In the as-sintered state, the test Fe alloy coupons were covered in a rough, greenish oxide layer, characterized to contain oxides of Mn in the case of Mn-containing coupons. This made them unfit for cell cytotoxicity testing and therefore this layer was removed. To this end, each coupon was manually ground to the desired P2500 finish under a constant flow of water, as shown in Table 8. A single coupon of each condition was subsequently polished to $<1\mu\text{m}$ for the purposes of material characterisation using scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDS). For the purposes of X-Ray Diffraction (XRD), grinding took place up to a grit of P1200.

In order to achieve the desired finish, grinding took place with sequentially finer grit SiC grinding paper (MetPrep®, UK) loaded onto a Struers® LaboSystem (Denmark), manual grinding machine with a running supply of water throughout the grinding and polishing processes. While wearing gloves, a starting grit of P400 grinding paper was used, this being chosen in order to remove the rough oxide layer having formed during the sintering process. Once the rough oxide layer had been removed, grit P600 grinding paper was loaded and used. This was followed by sequentially finer grits of P800, P1200 and finally, P2500. After each grinding step, the coupons were rinsed with 99% Isopropyl Alcohol (IPA) and kept immersed in fresh batches of 99% IPA until a finer grit paper was loaded. Immersion in 99% IPA was performed in order to prevent rusting of the coupons in between steps, since each grinding step necessitated exposing the coupons to water.

After the final grinding step using P2500 grinding paper, the coupons were submerged in a fresh batch of 99% IPA and ultrasonicated for five minutes. With ultrasonication complete, a final rinse with 99% IPA was performed before the coupons were dried using compressed air.

The coupons were then loaded into a vacuum desiccator for two hours, after which they were individually stored in sealed containers. Silica beads were added to the containers to act as a desiccating agent, preventing rusting. The finished coupons were documented to be of 21.8mm diameter and 4mm thickness as in Figure 2.

Table 8 – Table outlining the different finishes, corrosion states and acid treatment applied to the metallic coupons and pins.

Surface Finish	Materials	Purpose	Corrosion and Acid Etching – <i>In Vitro</i> Tests	Corrosion and Acid Etching – <i>In Vivo</i> Tests
P1200 Coupons	Test Fe alloy materials	XRD analysis	Uncorroded; Acid etched	-
P2500 Coupons	Test Fe alloy materials and SS316LVM	<i>In vitro</i> cytotoxicity	hFOB – Uncorroded; Acid etched MC3T3-E1 – Uncorroded and Pre-corroded; Acid etched	-
<1µm Coupons	Test Fe alloy materials	SEM imaging and EDS analysis	Uncorroded; Before and after acid etching	-
P2500 Pins	FeMn FeMn1Ag FeMn5Ag	<i>In vivo</i> biocompatibility	-	Uncorroded; Acid etched

3.1.4.1.2. Polishing

Polishing was only performed on those coupons that were to undergo material characterisation. After completion of grinding to a grit of P2500, the coupons were ultrasonicated for five minutes and subsequently rinsed, both steps being performed in 99% IPA. Polishing was performed manually and began with the use of a Struers® 6µm diamond

suspension on a Struers® MD-Dac polishing cloth. A second polishing step was then performed using a finer Struers® 3µm diamond suspension on a similar MD-Dac polishing cloth. The final polishing step made use of a Struers® OP-S suspension and a Struers® MD-Chem polishing cloth to achieve a finish of <1µm. In between each step and after the final polishing step, the samples were ultrasonicated for five minutes in 99% IPA. Similar to the ground coupons, these coupons were dried using compressed air and loaded into a vacuum desiccator for two hours. The coupons were then stored in sealed containers in the presence of silica beads as a desiccating agent.

3.1.4.2. Pin Preparation for *In Vivo* Experiments

For the purposes of *in vivo* experimentation, pins made from FeMn, FeMn1Ag and FeMn5Ag were fashioned from the previously sintered and ground Fe alloy coupons. The coupons were loaded into a lathe and machined to form cylindrical pins of 5mm x 2.05mm dimension as in Figure 3.

3.1.4.3. Acid Treatment of Test Iron Alloy Coupons and Pins

All Fe alloy coupons and pins were acid treated in order to remove MnO residue from their surfaces and within their pores. This was performed directly prior to material characterisation or sterilisation of the coupons and pins. This was done in order to prevent the re-accumulation of this product, as this acts as a passivation layer.

A 1M HCl solution was prepared from a stock 12M HCl solution, with 0.025M concentration of HMTA (Hexamethylenetetramine) added to this in order to act as a corrosion inhibitor. This solution was added to the dried coupons and pins and ultrasonication was immediately performed for five minutes. After ultrasonication, the coupons and pins were first rinsed with

de-ionised water to remove the acid solution, ultrasonicated in 99% IPA for a further five minutes and subsequently dried and loaded into a vacuum desiccator for two hours. Storage was not performed at this point, instead the coupons and pins were immediately characterised or used for *in vitro* cytotoxicity and *in vivo* biocompatibility tests respectively.

3.1.4.4. Material Characterisation and Imaging

All steps involving the characterisation of materials prior to their use in *in vitro* cytotoxicity and *in vivo* biocompatibility testing were performed with the assistance of Ms. Christabelle Tonna as part of her ongoing doctoral work.

Microscopic analysis of the metal surfaces was carried out using a Carl Zeiss® FE-SEM, Merlin Gemini II column (Germany) equipped with a Secondary Electron (SE) detector. Prior to imaging, gold sputter coating was performed for thirty seconds using an automatic sputter coater (Agar, USA). Subsequent EDS analyses was performed using an AMETEK® EDAX detector (USA), removing gold from the sputter coating from measurement.

Representative coupons were used for phase analysis using XRD. These samples were ground to a grit of P1200 using the method described in *Section 3.1.4.1.1 – Grinding*. They were then dried and scanned between 2θ angles of 30° to 100° in steps of 0.02° and at a scan rate of $1^\circ/\text{min}$ using a Bruker® D8 Advance (USA) diffractometer with a Bragg-Brentano setup. A copper X-ray source was used ($\text{Cu-}\alpha$, $\lambda = 0.141\text{nm}$) and the coupons were rotated at 15 rpm during the scan.

3.2. Cell Culture

3.2.1. Reagents, Materials and Equipment

A number of reagents, materials and equipment were necessary to perform cell culture techniques and *in vitro* cytotoxicity tests. These are listed in Tables 9, 10 and 11.

Table 9 – Reagents used during the performance of cell culture techniques and *in vitro* cytotoxicity tests

Reagent	Brand
Completed hFOB medium	Prepared fresh
Completed MC3T3-E1 medium	Prepared Fresh
Dulbecco's modified eagle medium/nutrient F12(DMEM-F12)	Gibco™, Thermo Fisher Scientific, USA
Foetal bovine serum (FBS)	Gibco™, Thermo Fisher Scientific, USA
G418 disulfate salt solution (50mg/ml)	Sigma-Aldrich®, USA
Minimum essential medium α (MEM α)	Gibco™, Thermo Fisher Scientific, USA
Penicillin-Streptomycin (5,000U/ml)	Gibco™, Thermo Fisher Scientific, USA
Phosphate buffered saline (PBS)	Sigma-Aldrich®, USA
CellTiter-Glo® luminescent cell viability assay	Promega, USA
Trypan blue solution (0.4%)	Invitrogen™, Thermo Fisher Scientific, USA
Trypsin (2.5%), without phenol red	Gibco™, Thermo Fisher Scientific, USA
Deionised water	
Ethanol	Gibco™, Thermo Fisher Scientific, USA
Liquid nitrogen	Multigas, Malta
Fe alloy coupons	
Stainless Steel 316LVM (SS316LVM)	Klein, Switzerland
Thermanox™	Nunc™, Thermo Fisher Scientific, USA
Glutaraldehyde (25%)	Acros Organics, USA
Hexamethyldisilazane	TCI, Japan
Sodium cacodylate trihydrate buffer pH 7.3 (0.1M and 0.2M)	Sigma-Aldrich®, USA

Table 10 – Materials used during the performance of cell culture techniques and *in vitro* cytotoxicity tests

<u>Material</u>	<u>Brand</u>
Conical centrifuge tubes	Corning®, USA
Microcentrifuge tubes	Corning®, USA
Cell culture flasks	Thermo Fisher Scientific, USA
Micropipettes	Gilson®, France
Serological pipettes	VWR International, USA
Cryovials	Corning®, USA
6-well plates	Corning®, USA
96-well, opaque-walled microplates	Corning®, USA
Cell counting slides	Invitrogen™, Thermo Fisher Scientific, USA

Table 11 – Equipment used during the performance of cell culture techniques and *in vitro* cytotoxicity tests

<u>Equipment</u>	<u>Brand</u>
Incubator	CellMate® CO ₂ Incubator – CO ₂ control, ESCO® Lifesciences, Singapore
Laminar flow cabinet	SafeFAST Elite, FASTER, DASIT Group, Italy
Automated cell counter	Countess™ 3 Automated Cell Counter, Invitrogen™, Thermo Fisher Scientific, USA
Centrifuge	Centrifuge 5810, Eppendorf®, Germany
Inverted light microscope	EVOS™ XL, Thermo Fisher Scientific, USA
Multimode microplate reader	Spark®, TECAN, Switzerland
Orbital plate shaker	Eppendorf®, USA
Autoclave	Boxer, UK
Fume hood	Industrial Loborum Ibérica, Portugal
Gold sputter-coater	Automatic Sputter Coater, Agar, USA
Scanning Electron Microscope	FE-SEM, Merlin Gemini II column, Carl Zeiss AG, Germany
Liquid nitrogen storage	Nitrogen Tank, Taylor Wharton, USA

3.2.2. Cell Lines

Two cell lines were chosen for the *in vitro* section of experiments. These were immortalised lines of human foetal osteoblasts (hFOB, ATCC®, CRL-11372™, USA), and murine (Mus musculus) pre-osteoblasts (MC3T3-E1 Subclone 4, ATCC®, CRL-2593™). The hFOB cell line in particular was chosen after consideration of the intended use of the eventual metal implant within humans. Both cell lines behave *in vitro* as osteoblasts, including the MC3T3-E1 cell line, which shows high differentiation towards an osteoblast cell lineage. In particular, this cell line was chosen in order to perform a biological replicate of the cytotoxicity tests performed using the hFOB cell line, as MC3T3-E1, coming from rodents, would allow for a more accurate correlation between the *in vitro* and *in vivo* tests on rats also undertaken in this project.

3.2.3. Culture Conditions

All procedures performed on the cell lines were performed under culture conditions and within a laminar flow hood (FASTER, DASIT Group, Italy), in order to prevent infections and ensure reliable results. MC3T3-E1 and hFOB cell lines require different culture conditions and therefore two separate incubators had to be used for the culture of these cell lines. The first was set at 34°C and 5% Carbon dioxide (CO₂) for the hFOB cell line and the second was set at 37°C and 5% CO₂ for the MC3T3-E1 cell line. Table 12 summarises the culture conditions for both.

Table 12 – The cell lines used for *in vitro* cytotoxicity tests, including their incubation temperature, medium constituents and seeding concentration.

Cell Line	Incubation Temperature (°C)	Medium	Medium Additives	Subculture Ratio
hFOB	34	Dulbecco's Modified Eagle Medium	10% FBS; 0.3mg/ml G418	1:4
MC3T3-E1 Subclone 4	37	Alpha Minimum Essential Medium	10% FBS; 50 I.U Penicillin; 50µg/ml Streptomycin	1:6

3.2.4. Basic Cell Culture Procedures

3.2.4.1. Thawing of Cell Lines

Both cell lines were obtained from storage in liquid nitrogen (Taylor Wharton, USA). The cell lines were rapidly thawed by placing the cryovials in a vessel containing water set at 34°C for the hFOB cell line and 37°C for the MC3T3-E1 cell line. The cryovial lids were not immersed in order keep them dry and prevent contamination with resultant infection. Once thawed, the cryovials were cleaned with 70% ethanol before they were opened and the contents rapidly transferred into 15ml conical centrifuge tubes (Corning®, USA) prepared to contain 5ml of medium appropriate for each cell line. This diluted the toxic cryoprotectant and prevented cellular injury.

Centrifugation was performed for five minutes at 1500rpm, or the equivalent of 435x gravity in a Centrifuge 5810, Eppendorf® (Germany), precipitating a pellet of cells at the bottom of each conical centrifuge tube. The supernatant was discarded and the remaining pellet of cells was resuspended in a fresh aliquot of 4ml of medium, the total content being transferred to a T25 cell culture flask. These were viewed under inverted light microscopy

using an Evos™ XL microscope (Thermo Fisher Scientific, USA), and placed in an incubator set for each cell line. The cells were viewed under inverted light microscopy twenty-four hours later in order to ensure their adherence to the flask's culture surface and also to ensure the absence of infection. Growth was followed regularly, with medium changes every three days as necessary until sub-confluence was reached. This was deemed to be 80% confluence and was the point at which subculture was performed as in *Section 3.2.4.3 – Cell Subculture*, with the only difference being that the cells were instead transferred in their entirety to a single 75cm² cell culture flask, until the next subculture procedure.

3.2.4.2. Renewal of Cell Culture Medium

Cell culture medium was changed every three days in accordance with ATCC® recommendation for both the hFOB and MC3T3-E1 cell lines. Before changing medium, the cells were observed under inverted light microscopy in order to ensure that cell growth was progressing as expected and that cells had not reached sub-confluence, in which case a subculture technique was instead performed. Examination for signs of infection was also performed at this time and would be indicated by the macroscopic presence of a cloudy yellow medium, as well as the visualisation of bacterial cells under inverted light microscopy.

Under the laminar flow hood, the flasks were opened and the old medium poured out into a receptive container, in order to be discarded afterwards. A fresh 15ml volume of completed medium was then pipetted into each of the T75 cell culture flasks. In the case of T25 cell culture flasks, 4ml volumes of medium were used instead. The flasks were then closed, removed from the laminar flow hood and viewed under inverted light microscopy. They were then placed within the incubator and the caps placed in vent position. The cells were left to

incubate until the next examination under inverted light microscopy, cell culture technique or cytotoxicity test.

3.2.4.3. Cell Subculture

Cell subculture was performed when cells reached sub-confluence. This point was taken to be a confluence of 80% and was chosen in order to allow cells growing in the log phase of growth to continue growing within this phase once transferred to new cell culture flasks. In order to confirm sub-confluence, the cell culture flasks containing cells were first removed from the incubator and examined macroscopically for signs of infection. Microscopic examination under inverted light microscopy was subsequently performed, looking for confluence and any signs of infection. Once the absence of infection and the achievement of sub-confluence were confirmed, the flasks were transferred to an appropriately prepared laminar flow hood, where a subculture technique was performed.

New T75 cell culture flasks were prepared and labelled with the following information: (1) Cell line; (2) Passage number; (3) Medium; (4) New flask number; (5) Previous flask number (in order to trace infections if any presented); (6) Date of subculture and (7) Researcher (LS).

The cell culture flasks containing cells were placed under the laminar flow hood and opened. The old medium was poured out into a receptive container in order to be discarded afterwards. A 3ml volume of PBS was pipetted over the flask's culture surface to rinse the surface and remove traces of old medium containing FBS. This volume was then discarded and replaced by a 3ml volume of 1x trypsin-EDTA. This second rinse ensured the removal of all traces of FBS, which would otherwise inactivate the fresh aliquot of trypsin. The volume of trypsin was then removed and a 3ml volume of warmed 1x trypsin (37°C) was pipetted over the culture surface of each of the cell culture flasks. The flasks' caps were replaced and the

flasks left inside the incubator at 37°C for five minutes, this time and temperature being chosen in order to ensure adequate trypsin activity over sufficient time. During this period, the caps were placed in vent position in order to ensure a continued supply of O₂ and CO₂.

Examination under inverted light microscopy was subsequently performed to ensure adequate detachment of cells from the flask's culture surface. A 3ml aliquot of fresh completed medium (containing FBS) was then added to each flask in order to de-activate the trypsin within and prevent unnecessary damage to cells. A pipette was used to draw up 5ml of the total of 6ml of fluid now within the flask and this volume was placed within an appropriately labelled 15ml conical centrifuge tube. A fresh pipette was then used to draw up a further 3ml of fresh medium and this volume was pipetted over the flask's culture surface. This ensured that all cells were then drawn up within the remaining 4ml present within the flask. This 4ml volume was added to the conical centrifuge tube for a total of 9ml of volume containing cells. The old and now empty cell culture flasks were subsequently discarded.

Centrifugation was performed for five minutes at 1500rpm (435x gravity), leaving a pellet of cells in the base of each conical centrifuge tube with a supernatant consisting of a mix of medium and inactivated trypsin. The supernatant was carefully poured out, ensuring not to agitate the tube and dislodge the cells resting at the bottom, as this would have resulted in their loss.

If cell counting was to be performed at this stage, the cells were re-suspended in a 1ml volume of fresh completed medium and counting performed as described in *Section 3.2.4.4 – Cell Counting*. Once counting was completed, the cells were used as necessary to seed the metallic test coupons and Thermanox™ coverslips for the subsequent cytotoxicity testing procedure, or re-suspended in 4ml or 6ml of medium respectively for hFOB and MC3T3-E1 cell lines. These were then split into appropriately prepared, fresh T75 cell culture flasks in a 1:4

ratio for the hFOB cell line and a 1:6 ratio for the MC3T3-E1 cell line. Fresh completed medium was added to each flask to make up a total of 15ml. Gentle agitation ensured adequate spread of medium containing cells over the whole of the flask's culture surface. The flasks were closed, examined under inverted light microscopy and placed within the incubator with the caps placed in vent position before closing the incubator. Finally, they were left for twenty-four hours before they were re-examined under inverted light microscopy to ensure adequate cell adherence and the absence of infection.

3.2.4.4. Cell Counting

Cell counting took place during cell subculture procedures and before seeding of cells onto the metallic test coupons and Thermanox™ coverslips in cytotoxicity tests. After trypsinisation and centrifugation of the cells as described in *Section 3.2.4.3 – Cell Subculture*, the supernatant was discarded and the cells re-suspended in a 1ml volume of fresh medium. A 20µl volume was then pipetted into a microcentrifuge tube (Corning®, USA) with a further 20µl volume of Trypan blue 0.4% (Invitrogen™, ThermoFisher Scientific, USA) being added. The resulting 40µl volume was mixed and 20µl of it removed and added to an appropriate cell counting slide. This was then loaded into the automated cell counter (Countess™ 3 Automated Cell Counter, Invitrogen™, Thermo Fisher Scientific, USA) or counted manually as in Equation 2 to find the total number of viable cells within the sample.

Equation 2 – The calculation performed during manual cell counting. The dilution factor represents the dilution with Trypan blue. As equal parts (20µl) of cell suspension and Trypan blue were added, the dilution factor was always two.

$$Cells/ml = \frac{Number\ of\ cells\ counted}{Number\ of\ squares\ counted} \times Dilution\ factor \times 10^4$$

3.3. Direct *In Vitro* Cytotoxicity Testing

3.3.1. Preparation and Setup of Cytotoxicity Tests

3.3.1.1. Sterilisation

Both as-ground and pre-corroded Fe alloy test metal coupons, along with SS316LVM coupons and Thermanox™ coverslips were sterilised immediately prior to their use in *in vitro* cytotoxicity tests. In order to ensure sterility, this procedure was performed in the laminar flow hood. The metallic coupons and Thermanox™ coverslips were each placed inside a sterile 6-well plate and 5ml of 70% ethanol added, this volume being able to cover all surfaces of the coupons and Thermanox™ coverslips. Each well was only loaded with a single metal coupon or Thermanox™ coverslip in order to ensure even ethanol contact on all surfaces. The 6-well plates were then left inside the laminar flow hood for a period of thirty minutes, after which the metal coupons and Thermanox™ coverslips were removed and placed inside freshly opened, sterile 6-well plates. This was performed using forceps sterilised in a 70% ethanol solution. The coupons were allowed to dry for thirty minutes, after which they were rinsed with PBS. A second rinse was then performed with fresh cell culture medium and the coupons and Thermanox™ coverslips were loaded into fresh, sterile 6-well plates. They were immediately used for cytotoxicity testing without storage periods, minimising the risk of contamination and infection.

3.3.1.2. Cytotoxicity Tests on As-Ground Coupons using an hFOB Cell Line

Direct cytotoxicity tests were performed over a period of four days, with cell viability testing using CellTiter-Glo® viability assay being performed after one, two and four days of hFOB cell exposure to test materials. In the first 6-well plate were placed three Thermanox™

coverslips and three SS316LVM coupons, as in Figure 6. A sterilised SS316LVM coupon was placed underneath each of the Thermanox™ coverslips, thus raising each coverslip to the same height within the well as the Fe-alloy and SS316LVM coupons. This ensured that the same height of cell-containing medium was present above all the test materials, including Thermanox™ coverslips, when these were seeded. This plate was labelled 'Control'.

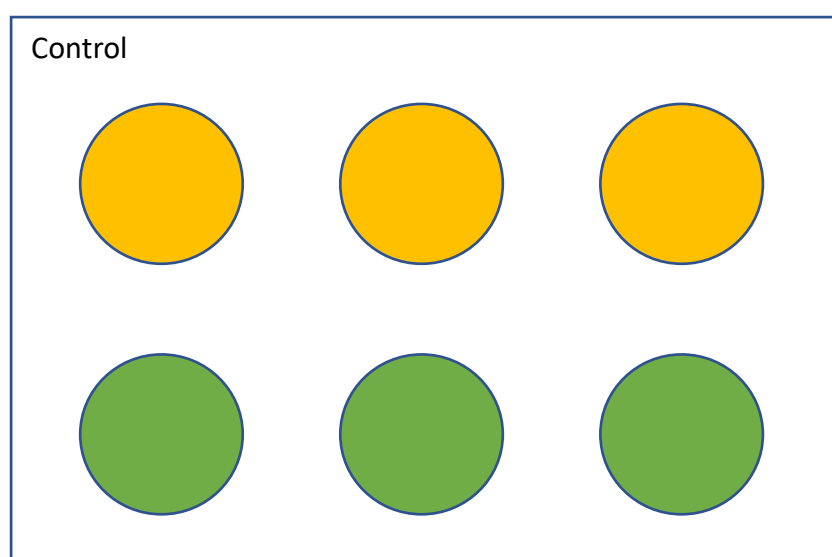


Figure 6 – Control 6-well plate containing the positive control specimens Thermanox™ (yellow) and SS316LVM (green).

A further four 6-well plates were then prepared for each day of testing. Each of these 6-well plates was set up to contain three as-ground, uncorroded coupons of a single Fe alloy test material, as demonstrated in Figure 7. Each plate was labelled according to the Fe alloy material it contained, the cell line being used and the day on which cell viability testing was to be performed. A final 6-well plate was used for each day of testing and was set up to contain a negative control well filled with medium only and no cells, as shown in Figure 8. This plate was labelled 'Medium'.

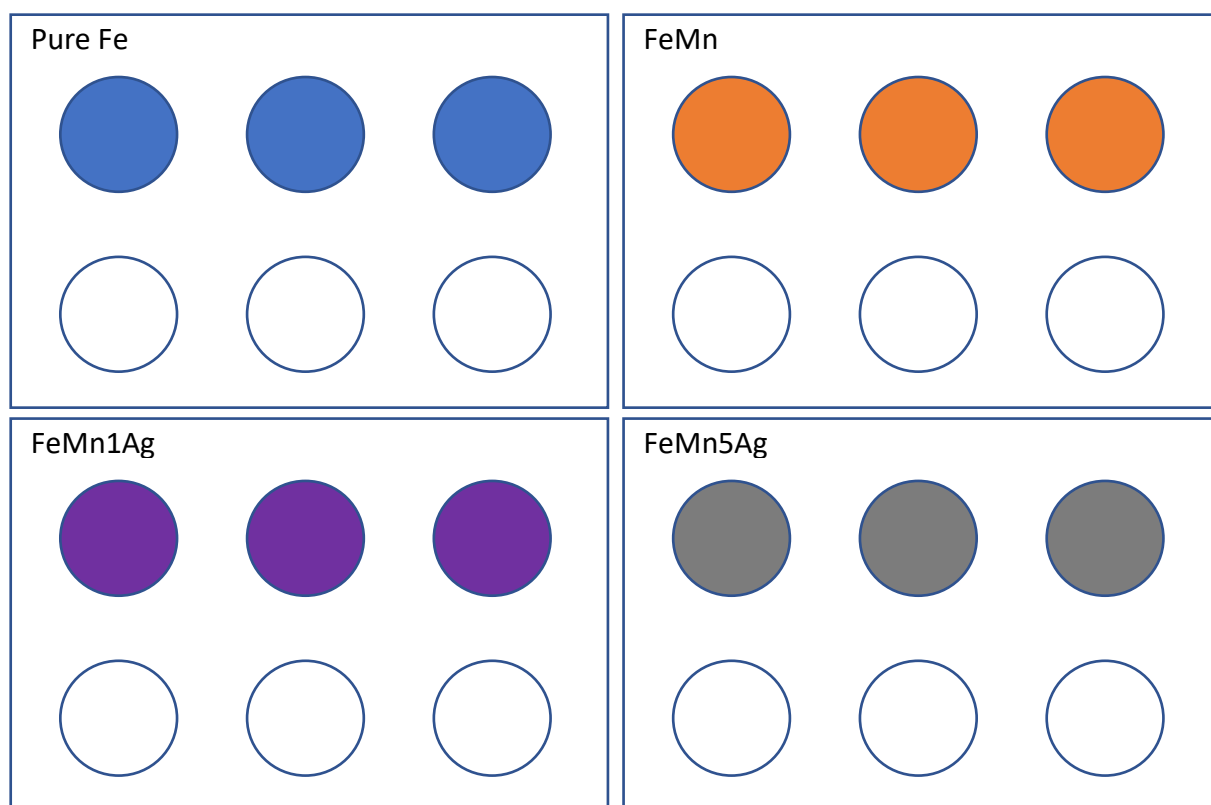


Figure 7 – Setup up 6-well plates for *in vitro* cytotoxicity tests using an hFOB cell line and as-ground, uncorroded coupons. Each 6-well plate contained three of either pure Fe (blue), FeMn (orange), FeMn1Ag (purple) or FeMn5Ag (grey) coupons. White wells were left empty.

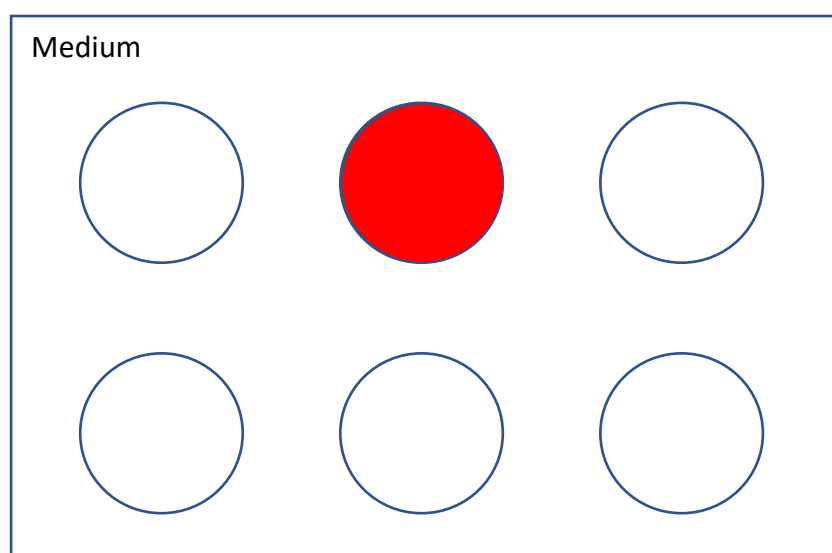


Figure 8 – Negative control 6-well plate with a single well filled with medium only (red). No cells were seeded into this well allowing a reading for background luminescence to be obtained. White wells were left empty

3.3.1.3. Biological Replicate on Pre-Corroded Coupons

After the first set of cytotoxicity tests using hFOB cells seeded onto as-ground, uncorroded coupons, a biological replicate was performed using an MC3T3-E1 cell line. This biological replicate was performed similarly to the tests using hFOB cells, save for some changes as indicated below;

1. After sterilisation, the test Fe alloy coupons were pre-corroded in medium for a total of three days. This was also performed for the SS316LVM² control coupons. After a three-day immersion period in cell culture medium, these coupons were rinsed twice in fresh cell culture medium before being seeded with cells for cytotoxicity testing.
2. A single as-ground, uncorroded Fe alloy coupon of each type (pure Fe, FeMn, FeMn1Ag and FeMn5Ag) was added to each testing day. This was done in order to be able to compare results that would have been obtained under the conditions of the first set of cytotoxicity tests using the hFOB cell line.

The reasons for performing this biological replicate were three-fold. The first was that assessing variability across cell lines would give more robust data regarding the toxicity of the Fe alloy materials in question. The second was that it was desirable to use rodent cells (MC3T3-E1) in order to be able to better link *in vitro* cytotoxicity with subsequent *in vivo* tests that were to be performed on rats. The third reason was to introduce the element of pre-corrosion.

² SS316LVM is, for all intents and purposes, immune to corrosion in this environment and over this time period.

The rate of corrosion of the Fe alloy coupons was expected to decrease after the first few days of exposure to medium, possibly due to the coupon becoming covered by insoluble corrosion products. This would subsequently decrease the amount of toxic, soluble corrosion products being released into the coupons' surrounding environments. It was suspected that this, in turn, would affect the level of cytotoxicity and viability of cells cultured in contact with these coupons. In addition, this would increase the similarity to the *in vivo* situation where the continued presence of osteoblasts and MSC would mean the continual seeding of the graft throughout the process of corrosion.

A setup similar to that used for the first set of cytotoxicity tests using as-ground coupons with an hFOB cell line was used. The positive control plate was set up as shown in Figure 6 and a negative control plate was also set up, as shown in Figure 8. In this case however, the SS316LVM coupons were pre-corroded for three days in medium prior to being seeded with cells, as was also performed for the test Fe alloy materials. The control plates were set up three times, once for each of days one, two and four of testing.

As for the test Fe alloy materials, the first three coupons within each 6-well plate had been pre-corroded in medium for three days prior to being seeded with MC3T3-E1 cells. In addition, an as-ground, uncorroded coupon of each Fe alloy material was placed in the fourth well of each 6-well plate and seeded simultaneously, as demonstrated in Figure 9. This final coupon of each Fe alloy material was sterilised separately from the pre-corroded coupons, immediately prior to its use in this set of tests. Each plate was labelled according to the Fe alloy material it contained, the cell line used and the day on which cell viability testing was to be performed.

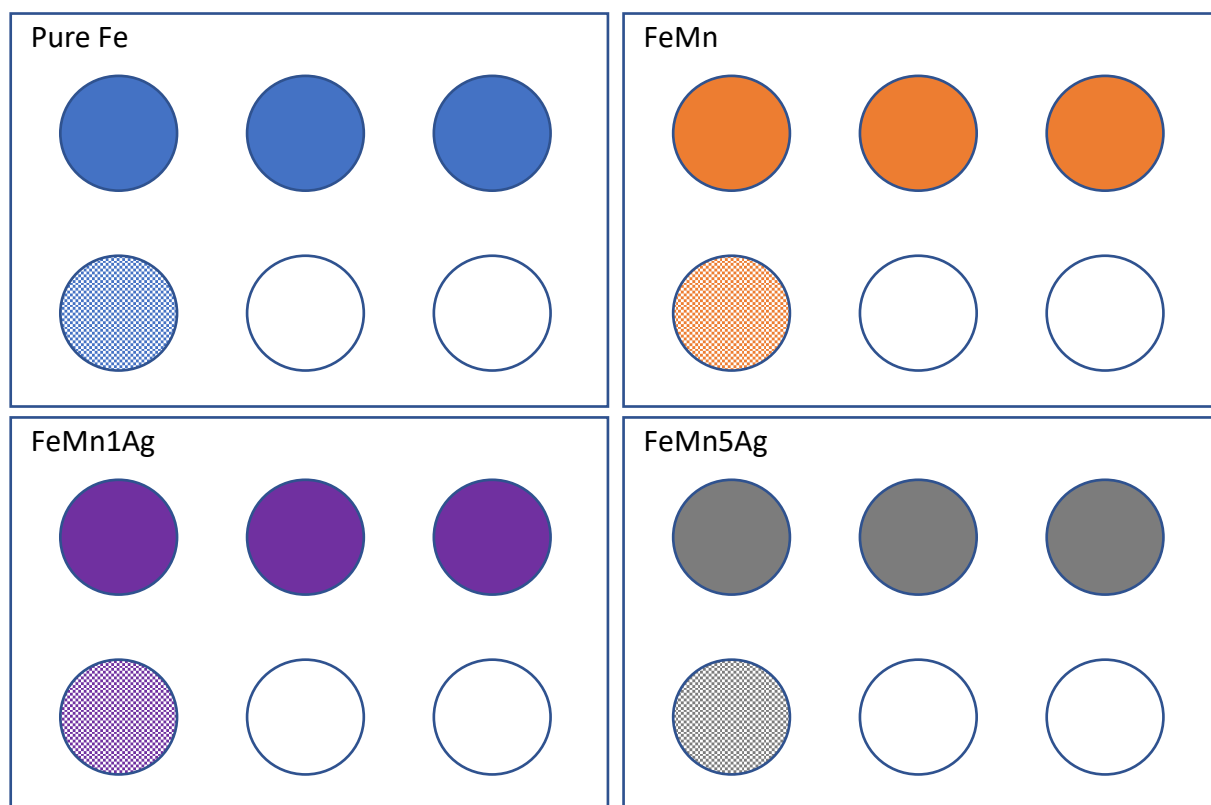


Figure 9 – Setup up 6-well plates for *in vitro* cytotoxicity tests using an MC3T3-E1 cell line. Each 6-well plate contained three pre-corroded coupons of either pure Fe (blue), FeMn (orange), FeMn1Ag (purple) or FeMn5Ag (grey) and a further coupon of the same as-ground, uncorroded material (cross-hatched). White wells were left empty.

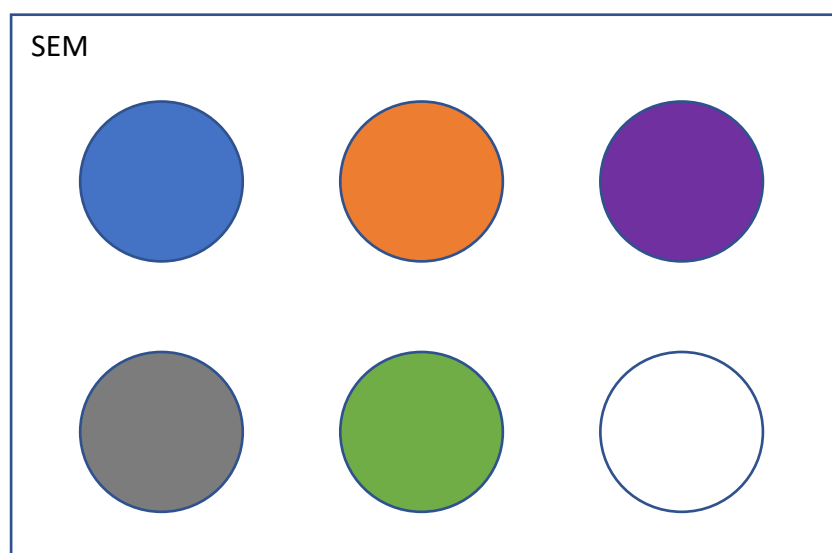


Figure 10 – Setup of 6-well plates with pre-corroded pure Fe (blue), FeMn (orange), FeMn1Ag (purple), FeMn5Ag (grey) and SS316LVM (green) coupons, destined for characterisation using SEM, EDS and reflective light microscopy imaging. White wells were left empty.

In this set of tests, a further 6-well plate was prepared for each day of testing. This plate contained one of each of the pre-corroded pure Fe, FeMn, FeMn1Ag, FeMn5Ag and SS316LVM coupons. These plates were labelled 'SEM' and would later be exposed to a cell-fixing protocol as in *Section 3.3.3.1 – Cell Fixing Protocol*. They would subsequently be gold sputter-coated to be examined using reflective light microscopy, SEM and EDS. The setup of this 6-well plate is shown in Figure 10.

3.3.1.4. Cell Seeding

Before the *in vitro* tests were performed, it was determined that any well containing a metal coupon or Thermanox™ coverslip would require a volume of 5ml of fluid for the material to be completely submerged. Prior to performing *in vitro* cytotoxicity tests with the corroding Fe alloy materials, trial runs with the non-toxic SS316LVM coupons and Thermanox™ coverslips were performed. In these trials, it was determined that a seeding concentration of 30,000 cells/ml for the hFOB cell line and 15,000 cells/ml for the MC3T3-E1 cell line would be required, as shown in Table 13. These concentrations allowed cells to continue to grow and multiply rapidly within the four-day testing period, without becoming over-confluent.

Table 13 – Passage and seeding concentration of cell lines used in *in vitro* cytotoxicity tests.

Cell Line	Seeding Concentration (Cells/ml)	Passage at Seeding
hFOB	30,000	30
MC3T3-E1 Subclone 4	15,000	25

Considering the seeding concentrations for both the hFOB and MC3T3-E1 cell lines, the total number of cells for each cytotoxicity test was determined. The cell lines were expanded sufficiently to ensure that all tests performed on the same cell line could be performed concurrently and with cells of the same passage. In addition, a minimum of three subculture techniques were performed before cytotoxicity testing was performed. This was done in order to ensure that cells were actively growing and were no longer affected by the freezing and thawing processes or the dimethyl sulfoxide (DMSO) cryoprotectant.

When the cell cultures were determined to have reached sub-confluence, a subculture procedure was initiated as described in *Section 3.2.4.3 – Cell Subculture*. Once trypsinisation and centrifugation had been completed, the cells were re-suspended in a 1ml volume of fresh medium and counted as described in *Section 3.2.4.4 – Cell Counting*. Using the calculated concentration of hFOB cells, a dilution was prepared to contain 30,000 cells/ml. This would ensure that 5ml would contain 150,000 hFOB cells. Seeding of each of the Fe alloy and SS316LVM coupons and Thermanox™ coverslips with 5ml of this dilution was then performed, ensuring the same number of cells within each test well. The absolute number of cells within each well was considered to be the most important variable since viability testing using CellTiter-Glo® reagent relies on the total amount of ATP present within the test sample. Differing volumes of the same concentration of cells would therefore produce unreliable results.

The same procedure was performed for the MC3T3-E1 cell line, instead preparing a dilution of 15,000 cells/ml which ensured the presence of 75,000 MC3T3-E1 cells within each 5ml volume. This was then used to seed each of the pre-corroded and uncorroded Fe alloy and SS316LVM coupons and Thermanox™ coverslips.

3.3.2. CellTiter-Glo® Test Procedure

3.3.2.1. CellTiter-Glo® Reagent Preparation

After the appropriate cell incubation time of between one and four days, CellTiter-Glo® viability testing was performed as per manufacturer guidelines. This cell viability assay comes packed from the manufacturer with two separate vials. The first is the CellTiter-Glo® Substrate, the second being the CellTiter-Glo® Buffer. These can be stored separately at -20°C until expiry.

Once needed, both the CellTiter-Glo® Substrate and Buffer were removed from storage at -20°C and allowed to thaw and equilibrate to room temperature. The contents of the 10ml CellTiter-Glo® Buffer vial was then poured into the vial containing the Substrate. This was mixed for a total of five minutes in order to create a homogenous mixture of the final CellTiter-Glo® Reagent. The prepared reagent was then allowed to rest to ensure equilibration to room temperature before use, as per manufacturer guidelines.

3.3.2.2. Trypsinisation Procedure

During the time taken by the reagent to equilibrate to room temperature, the 6-well plates with coupons and Thermanox™ coverslips seeded with cells were removed from the incubator and placed under the laminar flow hood. The medium present within each well was removed and the metal coupons and Thermanox™ coverslips transferred into fresh, sterile 6-well plates using sterilised forceps. While doing this, it was ensured that the coupons and coverslips were not rotated, thereby maintaining their correct orientation. They were then rinsed with PBS and subsequently rinsed for a second time with 1x trypsin. This ensured the removal of traces of medium containing FBS, which would inactivate the trypsin enzyme. The transfer of the metal coupons and Thermanox™ coverslips into fresh 6-well plates before trypsinisation was

also performed for a second reason. This prevented error from cells growing on the walls and bottoms of the old 6-well plates and not in direct contact with the Fe alloy coupons, SS316LVM coupons or Thermanox™ coverslips. Trypsin enzyme (1x) was then added at a volume of 5ml to each of the wells, in order to ensure that all surfaces were in contact with it. The 6-well plates were then transferred to the incubator and allowed to incubate at 37°C.

After an incubation time of five minutes, 3ml of medium was added to each of the wells in order to inactivate the trypsin enzyme. This volume was collected into a conical centrifuge tube and a further 3ml of medium used to rinse the coupons for a second time. This second volume was subsequently collected and transferred into the same conical centrifuge tube for a total volume of 11ml. Centrifugation then took place at 1500rpm (435x gravity) for five minutes. The resulting supernatant was discarded leaving any cells at the bottom of the conical centrifuge tube behind as a pellet. The cells were re-suspended in a 1ml volume of medium and 100µl of this volume pipetted into a further 200µl volume of medium. Of this 300µl volume, 100µl were transferred into a well of an opaque-walled 96-well plate. This dilution ensured that the total number of viable cells within the sample fell within the readable values of the CellTiter-Glo® assay and was information gained from trial assays performed on control specimens of SS316LVM and Thermanox™ before the final *in vitro* cytotoxicity tests.

To avoid confusion, the opaque-walled 96-well plates were set up similarly to the 6-well plates containing test materials, as depicted in Figure 11 for the hFOB cell line. This procedure was performed for all Fe alloy and SS316LVM coupons and Thermanox™ coverslips. In addition, a 100µl volume from the medium only well was transferred into one of the wells of the 96-well plate. The plate was then allowed to equilibrate to room temperature for a total of thirty minutes before the addition of 100µl of CellTiter-Glo® Reagent to each of the test wells. The plate was then placed in an orbital plate shaker (Eppendorf®, USA) for two minutes

and subsequently allowed to incubate at room temperature for a further ten minutes in order to allow cell lysis and stabilisation of the luminescent signal. The resulting luminescence signals were read using a Spark[®] multimode microplate reader (TECAN, Switzerland).

In the case of cytotoxicity tests with MC3T3-E1 cells, a similarly set up opaque-walled, 96-well plate was used. Here, each Fe alloy material had a fourth well for the as-ground, uncorroded coupon in addition to the three pre-corroded coupons, as shown in Figure 12.

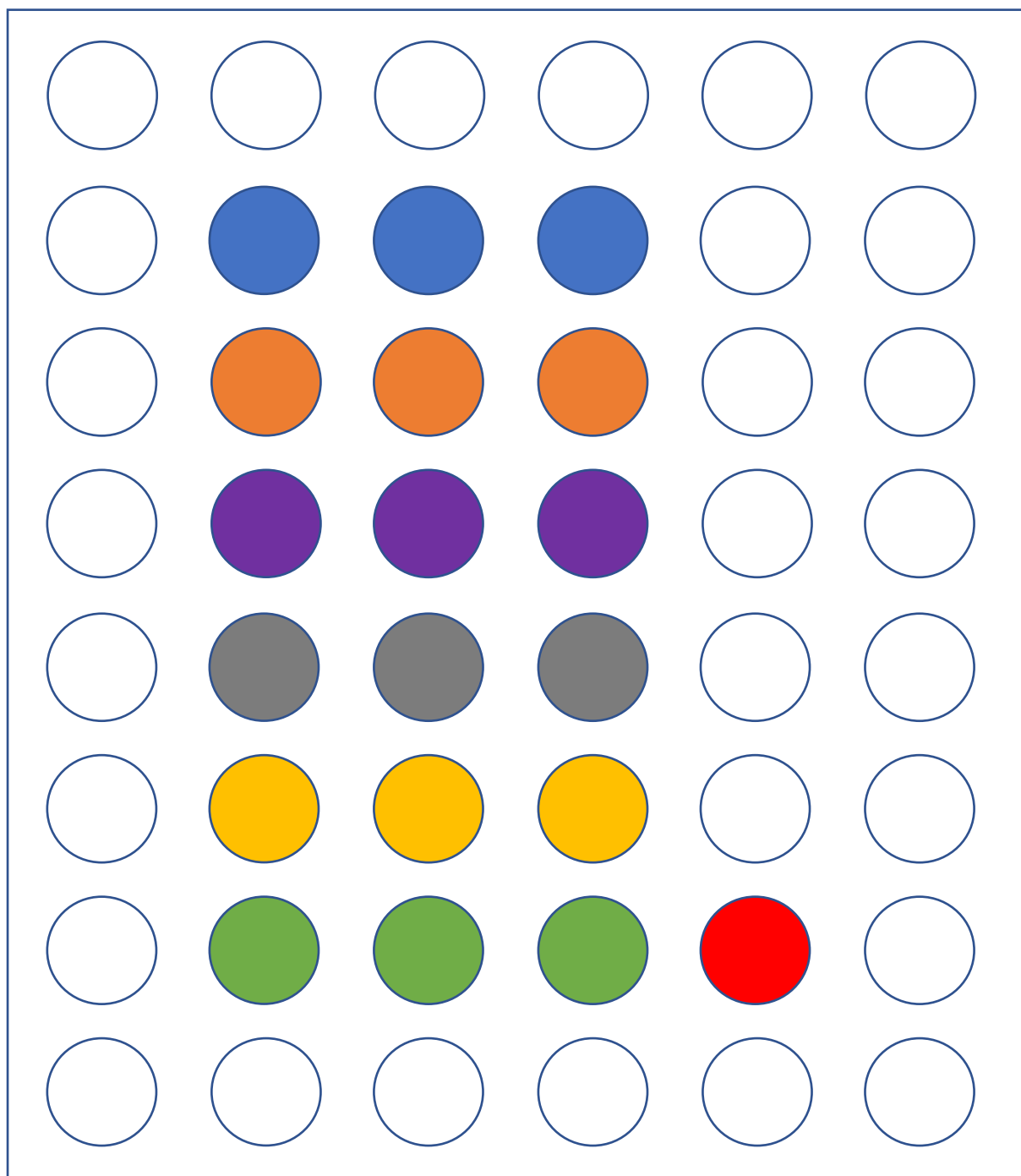


Figure 11 – Half of an opaque-walled, 96-well plate showing the setup used for the CellTiter-Glo® assay with the hFOB cell line. To avoid confusion, the setup is similar to that of the 6-well plates and shows uncorroded pure Fe (blue), FeMn (orange), FeMn1Ag (purple), FeMn5Ag (grey), Thermanox™ (yellow), SS316LVM (green) and medium only (red). White wells were left empty.

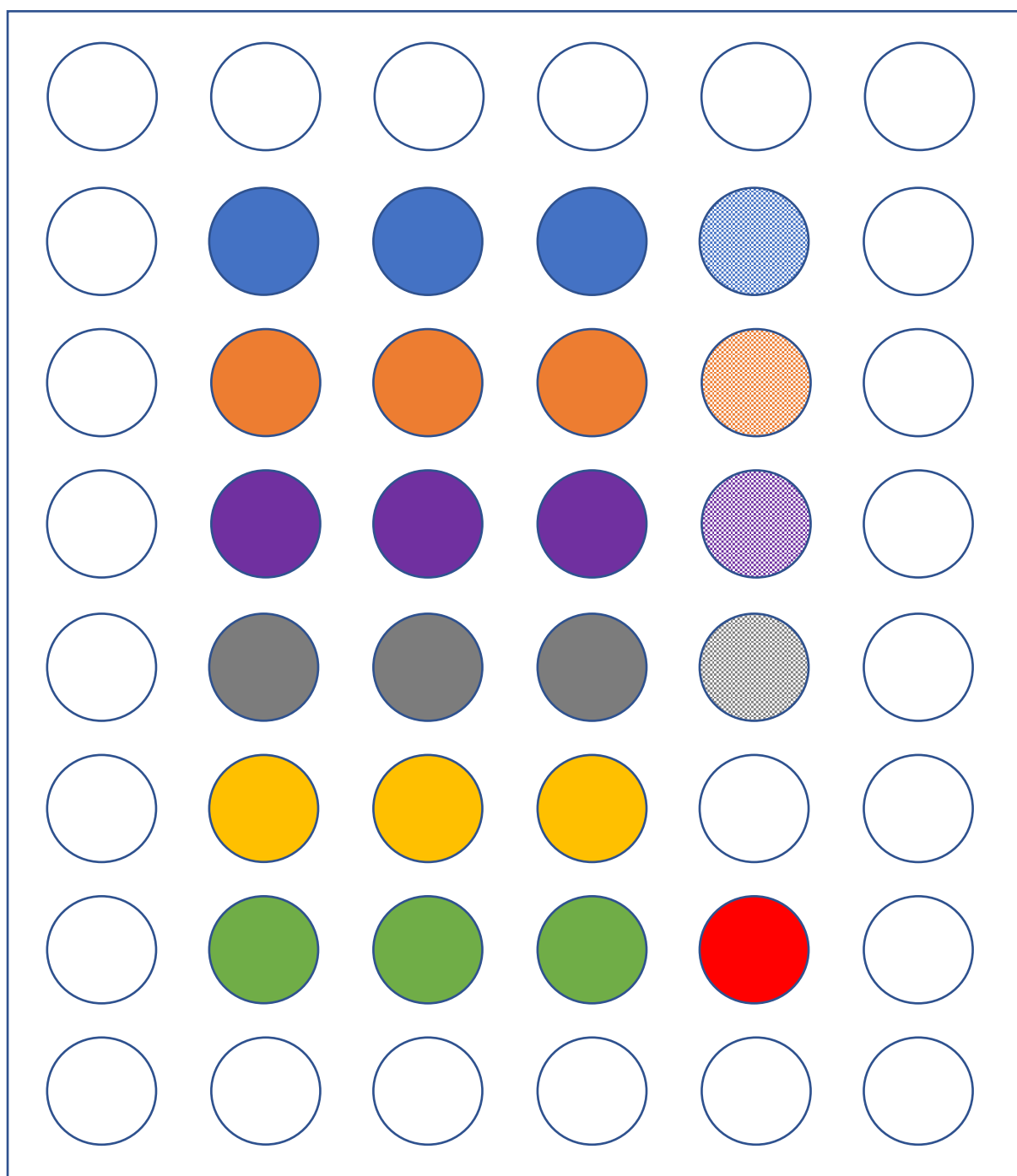


Figure 12 – Half of an opaque-walled, 96-well plate showing the setup used for the CellTiter-Glo® assay with the MC3T3-E1 cell line. To avoid confusion, the setup is similar to that of the 6-well plates and shows corroded pure Fe (blue), FeMn (orange), FeMn1Ag (purple), FeMn5Ag (grey), Thermanox™ (yellow), SS316LVM (green) and medium only (red). A final fourth as-ground, uncorroded coupon of each Fe alloy material was also tested and is depicted as the cross-hatched well in each row. White wells were left empty.

3.3.3. Characterisation and Imaging of Seeded Coupons

3.3.3.1. Cell Fixing Protocol

The metal coupons seeded with MC3T3-E1 cells to be viewed under reflective light microscopy and SEM were removed from the 6-well plate and submitted to a cell fixing and dehydration protocol. Each coupon was individually removed from its well, rinsed with PBS and placed in a similar position within a fresh 6-well plate. A 5ml volume of a cell fixing solution containing 50% v/v of 0.2M Sodium Cacodylate buffer pH 7.3, 40% v/v distilled water and 10% v/v of 25% Glutaraldehyde was added to each well. This solution was left for ten minutes, after which it was removed and replaced with 5ml of 0.1M Sodium Cacodylate buffer pH 7.3. This was left for ten minutes before being removed. Dehydration was achieved by adding 25% aqueous ethanol for ten minutes, this solution being replaced with 50% aqueous ethanol. At ten minute intervals, the ethanol solution was replaced by sequentially more concentrated solutions (60%, 70%, 80%, 90%), ending with 100% ethanol. After a further ten-minute interval, the ethanol was removed and Hexamethyldisilazane was added in a fume hood for a final ten minutes. This solution was subsequently removed and the coupons left to dry in the fume hood for a total of one hour. The coupons were then viewed under reflective light microscopy and SEM.

3.3.3.2. Imaging and EDS Examination

This procedure was performed with the assistance of Ms. Christabelle Tonna, as part of her ongoing doctoral work. The coupons were sputter-coated with gold for thirty seconds using an automatic sputter coater (Agar, USA).

In order to view the cells on the surfaces of the coupons, a reflective light microscope was used (Zeiss Axioscope 5, Carl Zeiss AG, Germany). Photographs were taken to provide a visual representation of any cell growth on the coupons' surfaces.

SEM imaging was performed on all metal coupons (Fe alloy and SS316LVM) subjected to a cell fixing procedure. This was performed using the same Carl Zeiss FE-SEM, Merlin Gemini II column microscope (Carl Zeiss AG, Germany) equipped with a Secondary Electron (SE) detector. EDS analysis was also carried out during the same procedure using an AMETEK EDAX detector (USA), removing gold from the sputter coating from measurement. This was performed in order to identify the predominant corrosion products present on the surfaces of the coupons.

3.3.4. Statistical Analysis

All statistical analyses were performed using the SPSS® 29.0 software platform (IBM® Corp., USA) and graphs were plotted on Origin 2021 (OriginLab®, USA). A two-way ANOVA model with interaction analysis was performed for each cell line, in order to test whether luminescence varied significantly in relation to the tested material, the day on which the cell viability assay was performed, and also whether these latter variables interacted significantly with each other.

In the case of the MC3T3-E1 cell line, a three-way ANOVA model with interaction analysis was performed in the first instance to determine whether pre-corrosion significantly affected the luminescence signals obtained.

3.4. *In Vivo* Biocompatibility Tests

In order to understand the *in vivo* biocompatibility of the Fe alloy materials under study, an experiment was designed wherein pins were fashioned from the same Fe alloy materials, as described in *Section 3.1.4.2 – Pin Preparation for In Vivo Experiments*, and implanted into rats. These were planned to be left *in vivo* for a period of six months before the rats would be euthanised and the pins explanted for characterisation.

As pure Fe is already known to be biocompatible *in vivo*, pins made from this material were not fashioned, ethical approval instead being gained for *in vivo* testing using FeMn (control material), FeMn1Ag and FeMn5Ag materials. A total of twenty-four pins were made and implanted into twenty-four individual rats. A key was kept to ensure that each rat had a known material implanted. The rats chosen for this experiment were GAERS rats as these were the most easily available. This rat is often used in epilepsy experiments and there should be no effect on biocompatibility results. The rats were kept separated from each other in standard housing cages in order to ensure that there was no interference between rats and that each rat had an implant of known composition. All *in vivo* techniques were performed with the assistance and under the supervision of Prof. Pierre Schembri Wismayer.

3.4.1. Ethical Approval

Ethical approval was sought and gained for the *in vivo* portion of this project. To this end, an application was submitted to the Department of Anatomy's Faculty Research Ethics Committee (FREC) within the University of Malta and reviewed by the Joint FREC Animal Research Sectoral Subcommittee (JFARRS). Approval for *in vivo* tests was granted on the 8th of March 2021, with *in vivo* tests being performed thereafter.

3.4.2. Reagents, Materials and Equipment

A number of reagents, materials and equipment were required for the performance of *in vivo* biocompatibility tests. These are listed in Tables 14, 15 and 16.

Table 14 – Reagents used during *in vivo* biocompatibility tests.

<u>Reagent</u>	<u>Brand</u>
Fe alloy pins	
Ethanol	Gibco™, Thermo Fisher Scientific, USA
Isoflurane	Sigma Aldrich®, USA
Chloramphenicol 1% w/w ointment	URSAPHARM, Germany
Formaldehyde	Sigma Aldrich®, USA
Formic acid	Sigma Aldrich®, USA

Table 15 – Materials used during *in vivo* biocompatibility tests.

<u>Material</u>	<u>Brand</u>
Sutures	Polypropylene 4/0, SMI, Belgium

Table 16 – Equipment used during *in vivo* biocompatibility tests

<u>Equipment</u>	<u>Brand</u>
Anaesthetic chamber	Sliding top chamber, Kent Scientific®, UK
Anaesthetic delivery system	SomnoSuite®, Kent Scientific®, UK
Anaesthetic mask	Low-profile anaesthesia mask, Kent Scientific®, UK
Surgical pad	SurgiSuite®, Kent Scientific®, UK
Surgical Instruments	Kent Scientific®, UK
Microdrill	OmniDrill35, World Precision Instruments, USA
Histological microscope	AxioStar Plus Binocular Microscope, Carl Zeiss AG, Germany
Manual rotary microtome	RM2235, Leica, USA
Tissue Processor	Vacuum Tissue Processor MTM-II, SLEE, Germany
Gold sputter-coater	Automatic Sputter Coater, Agar, USA
Scanning Electron Microscope	FE-SEM, Merlin Gemini II column, Carl Zeiss AG, Germany

3.4.3. Surgical Implantation

3.4.3.1. Sterilisation of Iron Alloy Pins

Before implantation, all Fe alloy pins to be implanted were sterilised by allowing them to rest in 70% ethanol for ten minutes. They were then removed from the ethanol solution and set on a sterile mat to dry for a further ten minutes before implantation. Each pin was sterilised individually and immediately prior to it being used in the implantation procedure.

3.4.3.2. Induction and Maintenance of Anaesthesia

Anaesthesia was induced and maintained using Isoflurane, a volatile anaesthetic agent. In order to induce anaesthesia, the rats were individually set in sliding top chambers obtained from Kent Scientific® (UK). A SomnoSuite® (Kent Scientific®, UK) anaesthetic system was used to deliver 1-5% Isoflurane in air, into the chamber containing the rat. Once anaesthesia had been induced, the rats were removed from the sliding top chambers and quickly set out on a SurgiSuite® operating platform (Kent Scientific®). Isoflurane delivery continued through a nose cone at 1-3% in air, using the same SomnoSuite® anaesthetic system. Depth of anaesthesia was monitored both before and during the operative procedure using the 'toe pinch' technique. The anaesthetic dose was changed accordingly during the course of the procedure to ensure that the rat did not experience any pain.

3.4.3.3. Operative Implantation of Iron Alloy Pins and Post-Operative Monitoring

Once anaesthesia had been induced and was deemed to be adequate, the rats were individually placed in a right lateral decubitus position on the SurgiSuite® operating platform. This was set up with adequate lighting to ensure unimpeded vision during the operative procedure. Sterile instruments were also set out on the operative platform with sterile surgical sutures being opened as needed by an assistant during the procedure.

Sterile gloves were worn and the rat's tail was sterilised using 70% ethanol. The tail was palpated and a minimally sized incision was placed laterally, over a single vertebra in the base of the tail. After incising the skin, soft tissues were dissected both sharply and bluntly to ensure minimal damage to these structures, which included tendons. With bone exposed, the OmniDrill35 Micro Drill (World Precision Instruments, USA) was used to create a defect

perpendicular to the long axis of the vertebra. This defect was made to accommodate the Fe alloy pin as snugly as possible and the implant was press-fitted into the created defect, as shown in Figure 13.

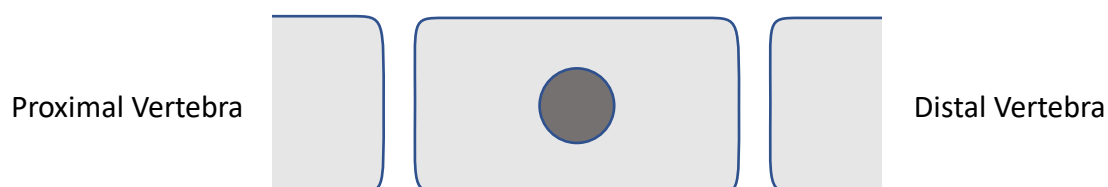


Figure 13 – Diagram indicating the positioning of the implanted pin within a vertebra. The central dark grey circle identifies the 2.05mm diameter surface of the pin which was inserted across the width of the vertebra.

Skin and soft tissues were then closed with polypropylene 4/0 (SMI, Belgium) interrupted sutures and Chloramphenicol 1% w/w ointment (URSAPHARM, Germany) was applied over the incision wound to help prevent infection. Anaesthetic agent delivery was stopped at this point and the rat was placed within its cage to be monitored while it recovered from anaesthesia.

After the operative procedure, the rats were left in their own separate cages for a period of six months, before planned euthanasia was performed. During this period, the rats were monitored for signs of stress and ill-health which may have indicated the implants' poor biocompatibility.

3.4.4. Examination of the Iron Alloy Pins and Rats

3.4.4.1. Euthanasia

After a six-month monitoring period, the rats were euthanised in order to be able to image and characterise the implants. Euthanasia was performed by individually placing the rats in a

closed container and administering a supply of CO₂. This ensured a quick and painless euthanasia procedure wherein the rats did not suffer unnecessarily.

3.4.4.2. Computed Tomography and Magnetic Resonance Imaging

All of the rats had their tails removed and numbered with indelible marker to ensure that the implant within each of the tails was known. These tail segments, which included the operated vertebrae, were then placed in order into a cardboard tube and this placed within a computed tomography (CT) scanner for imaging. In addition to this, some of the rat bodies were imaged using MRI, with specific sequences employed to detect accumulation of Fe within internal organs.

3.4.4.3. Explantation and Histological Examination of the Iron alloy Pins

To allow for histological examination of the tails, each was cut, keeping only the section with the implanted pin. The skin was removed, exposing the tissues underneath and each specimen was subsequently fixed in a bath of formaldehyde for forty-eight hours. A decalcification procedure was subsequently performed by placing the tails in formic acid. Alternate day replacement of the acid for a total of two to three weeks was performed. Dehydration, clearing and paraffin impregnation of the specimens was then performed with the help of an automated Vacuum Tissue Processor MTM-II (SLEE, Germany). Fixed specimens were sectioned using a manual rotary microtome (RM2235, Leica, USA) and imaged using a microscope (AxioStar Plus Binocular Microscope, Carl Zeiss AG, Germany). This procedure was also performed on control vertebrae taken from the same rats but not having an implant sited, as well as vertebrae with pins that had been lost. This was performed in order to be able to compare histological findings between implanted and non-implanted bone. Histological

examination was performed with the help of Prof. Pierre Schembri Wismayer and Mr. William Atanasio.

3.4.4.4. Characterisation of the Explanted Pins

Characterisation of the explanted pins was performed with the assistance of Ms. Christabelle Tonna, as part of her ongoing doctoral work.

Prior to characterisation, the explanted pins were gold sputter-coated for thirty seconds using an automatic sputter coater (Agar, USA). The explanted pins were then subjected to metallographic analysis via SEM using a Carl Zeiss FE-SEM, Merlin Gemini II column microscope (Carl Zeiss AG, Germany) equipped with a Secondary Electron detector, as was performed prior to implantation. EDS analysis was also performed using an AMETEK-EDAX detector, removing gold from the sputter coating from measurement. XRD analysis was performed between 2θ angles of 30° to 100° in steps of 0.02° and at a scan rate of $1^\circ/\text{min}$ using a Bruker® D8 Advance (USA) diffractometer with a Bragg-Brentano setup. A copper X-ray source was used ($\text{Cu-K}\alpha$, $\lambda = 0.141\text{nm}$) and the coupons were rotated at 15 rpm during the scan.

The pins were also cold-mounted in epoxy and one end was ground to a P2500 finish, exposing the material underneath the corroded surface. This was done in order to be able to examine the boundary between the pins and surrounding bone. Similar characterisation was performed as before with SEM and EDS.

Results

4.1. Metallographic Analysis of Pre-Test Iron Alloy Materials

After the sintering process, all of the Mn-containing coupons were found to be covered in a greenish, rough oxide layer, making them unsuitable to be used in either *in vitro* cytotoxicity or *in vivo* biocompatibility tests. This was also found on the pure Fe coupons, but to a lesser extent. This layer was removed through a grinding process, with characterisation performed for each material after a further polishing step, as described in *Section 3.1.4.1 – Surface Preparation*.

4.1.1. Characterisation of Polished Iron Alloy Coupons and Pins Before Acid Etching

4.1.1.1. Pure Iron

Directly after the polishing procedure, Fe alloy coupons were subjected to examination via SEM, with EDS also being performed. As is seen in Figure 14, SEM imaging of pure Fe showed a generally microporous structure with the majority of pores being smaller than 50µm in size. Also seen were pores of larger size, however these were much fewer in number and did not exceed a maximum size of 110µm. EDS analysis of the surface of a representative pure Fe coupon (marked as '1' in Figure 14) showed specifically high amounts of Fe, as was expected. Similar analysis of the pore regions of the pure Fe coupon instead showed large quantities of O, with Al and Si also being represented in significant amounts. These results are shown in Tables 17.

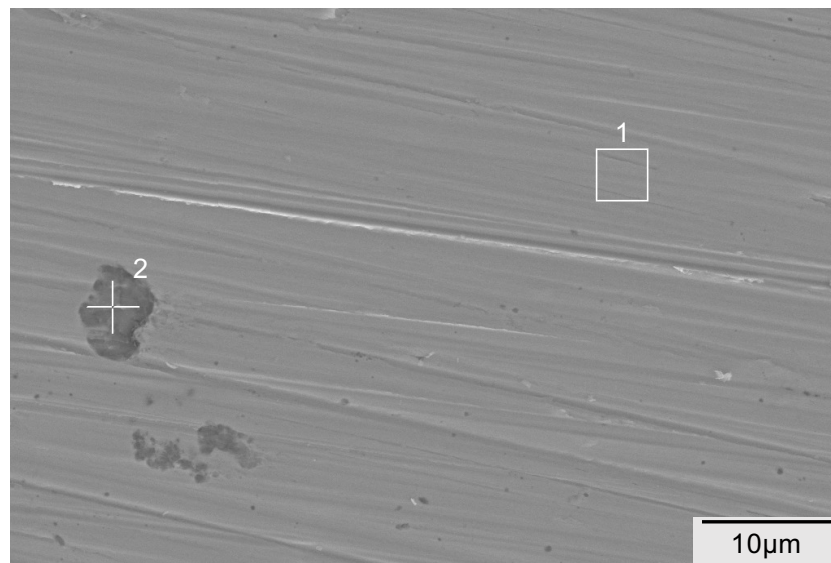


Figure 14 – SEM imaging of a pure Fe coupon showing micropores on its surface. EDS analyses of the coupon’s surface (1) and pores (2) were performed.

Table 17 – EDS analyses of the surface (1) and pores (2) of a pure Fe coupon showing the weight percentages of the detected elements.

Element	Fe	O	C	Mn	Mg	Al	Si	Ca	Ti	V
%Wt – 1	91.20	2.68	6.12	-	-	-	-	-	-	-
%Wt – 2	4.19	47.98	4.24	2.30	2.05	9.12	14.36	6.35	7.77	1.21

4.1.1.2. Iron-Manganese

SEM images of FeMn show a similar structure to that seen in pure Fe. The porous structure was noted to be microporous with the majority of pores being of a size below 50µm, as seen in Figure 15. Only a small number of pores were noted to be larger than this, however none of these exceeded 110µm. FeMn coupons were also noted to have more visible pores on their surfaces than did pure Fe coupons.

EDS analysis of the surface of a representative FeMn coupon showed the presence of small amounts of O, C and N, likely to be contaminants from the handling of the coupons during the grinding and polishing processes. Having excluded these elements, the ratio of Fe to Mn by weight, was calculated to be 65:35, as seen in Table 18.

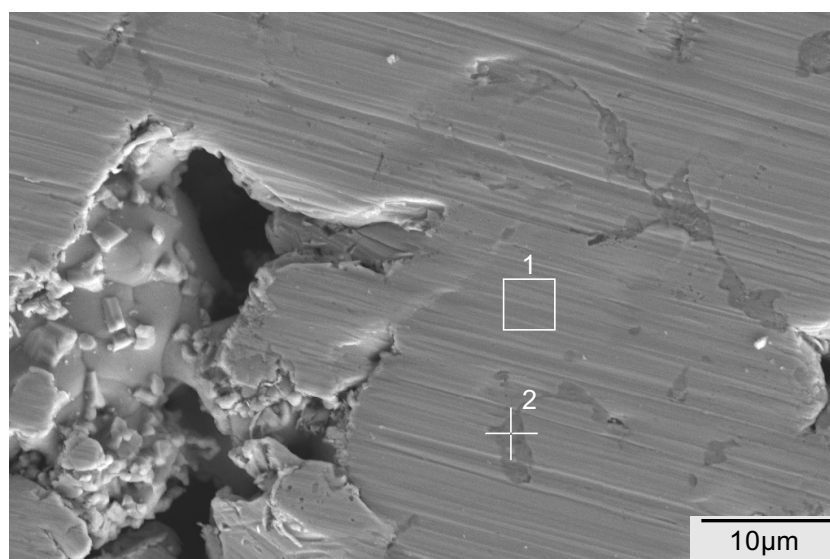


Figure 15 – SEM imaging of an FeMn coupon showing micropores on its surface as well as the presence of a macropore. EDS analyses of the coupon’s surface (1) and pores (2) were performed.

Table 18 – EDS analyses of the surface (1) and pores (2) of an FeMn coupon showing the weight percentages of the detected elements.

Element	Fe	Mn	O	C	N	Mg	Al
%Wt – 1	57.76	30.73	2.00	5.39	4.12	-	-
%Wt – 2	-	59.30	33.91	4.45	-	00.60	1.73

4.1.1.3. Iron-Manganese-Silver

SEM imaging with EDS analysis was also performed for both of the Ag-containing FeMn coupons. Their structure on SEM imaging is shown in Figure 16 and is similar to that seen for both pure Fe and FeMn. The majority of pores were below 50µm in size with only a few reaching sizes closer to 110µm. As was found to be the case for FeMn coupons, Ag-containing FeMn coupons were also seen to have more pores than the pure Fe coupons.

Striking, bright clumps were also noted to be present on both the surfaces of the FeMn1Ag and FeMn5Ag coupons, as part of their structures. These were present in greater number in the FeMn5Ag coupons, with EDS characterising these clumps to contain large amounts of Ag, as seen in Table 19. The clumps were discrete, with EDS also showing the absence of Ag within the remainder of the surface of the coupons. This suggested that Ag was only present within these brighter areas.

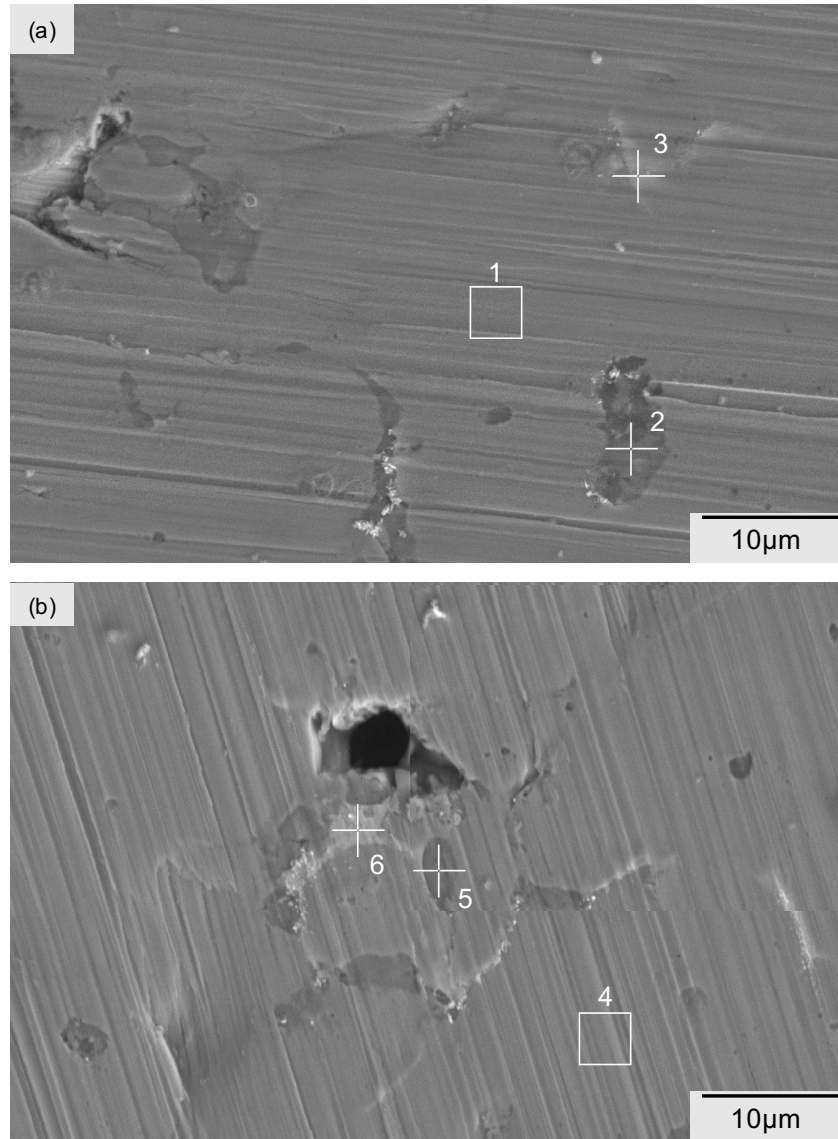


Figure 16 – SEM imaging of an FeMn1Ag coupon (a), showing micropores on its surface. EDS analyses of the coupon's surface (1), pores (2) and bright clumps (3) were performed. SEM imaging of an FeMn5Ag coupon (b) was also performed, alongside EDS analyses of its surface (4), pores (5) and bright clumps (6).

Table 19 – EDS analyses of the surfaces (1, 4), pores (2, 5) and bright areas (3, 6) of FeMn1Ag (1-3) and FeMn5Ag (4-6) coupons, showing the weight percentages of the detected elements.

Element	Fe	Mn	Ag	O	C	N	Al	Si	Mg
%Wt – 1	58.32	30.55	-	1.97	5.22	-	-	-	-
%Wt – 2	-	61.16	-	32.62	4.19	-	1.43	-	0.61
%Wt – 3	2.51	6.06	73.24	5.78	9.65	2.19	-	0.16	0.42
%Wt – 4	56.43	28.16	-	1.94	9.60	3.88	-	-	-
%Wt – 5	-	45.95	-	30.44	23.61	-	-	-	-
%Wt – 6	-	6.56	62.32	3.59	27.22	-	-	-	0.31

4.1.2. Characterisation of Polished Iron Alloy Coupons and Pins

After Acid Etching

As can be seen in *Section 4.1.1 – Characterisation of Polished Iron Alloy Coupons and Pins Before Acid Etching*, the micropores of all of the coupons of every material were filled with a substance which was characterised via EDS to have large amounts of O in the case of pure Fe. In the case of Mn-containing materials (FeMn, FeMn1Ag and FeMn5Ag), Mn and O were the predominant elements present. In view of this, an acid etching procedure was devised and performed as in *Section 3.1.4.3 – Acid Treatment of Test Iron Alloy Coupons and Pins*. The goal of this procedure was to remove the Mn oxide which had formed within the porous structure of the Mn-containing coupons during the sintering process. This was performed as it was likely that these oxides would affect the corrosion rate of these materials in an unknown manner. In order to document the effect of the acid etching procedure on the coupons, characterisation was also performed after the etching process had been performed.

4.1.2.1. Pure Iron

The pure Fe coupons remained unchanged by the acid etching procedure. SEM imaging as shown in Figure 17, as well as EDS analyses as shown in Table 20, showed no difference in structure or composition of both the coupon's surface and pores.

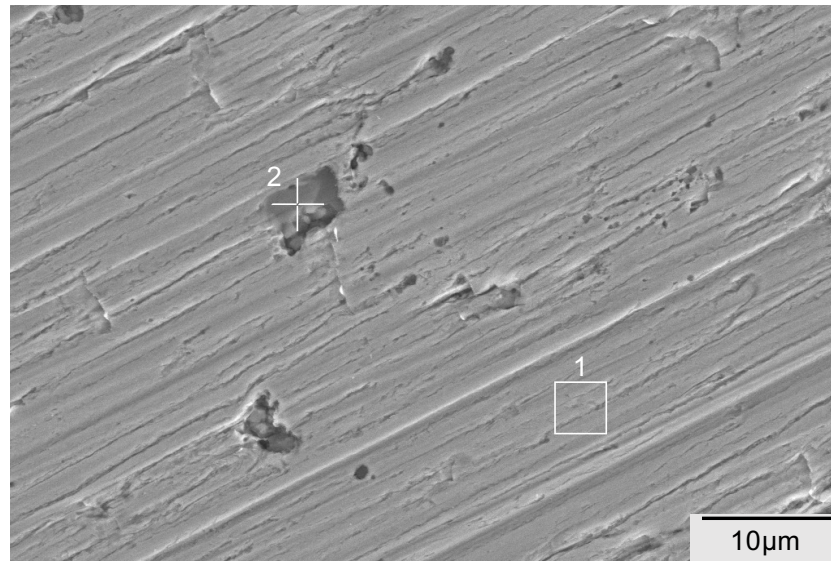


Figure 17 – SEM imaging of a pure Fe coupon after the acid etching procedure. The structure remained unchanged, with EDS analyses of the coupon's surface (1) and pores (2) yielding results similar to those from before acid treatment.

Table 20 – EDS analyses of the surface (1) and pores (2) of a pure Fe coupon after the acid etching procedure, showing the weight percentages of the detected elements.

Element	Fe	O	C	Mn	Mg	Al	Si	Ca	Ti	V
%Wt – 1	90.08	3.65	6.27	-	-	-	-	-	-	-
%Wt – 2	3.73	47.60	-	7.81	6.82	17.88	2.23	0.55	10.92	2.46

4.1.2.2. Iron-Manganese

The acid etching procedure had no effect on the porous structure of the FeMn coupons, with SEM imaging revealing similar structures both before and after acid treatment. Ag was also still noted to be dispersed as discrete clumps. A clear difference was however noted within the pores, acid treatment having cleared them of Mn oxide that was previously present. This is easily appreciated on both lower magnification SEM imaging as seen in Figure 18, as well as higher magnification imaging as seen in Figure 19.

EDS analysis after acid treatment showed significantly lower amounts of O and Mn within the pores of the coupon. The ratio of Fe to Mn of 65:35 had been maintained throughout the acid etching procedure and was clearly demonstrated on both the surface and pores of the coupon, as shown in Table 21.

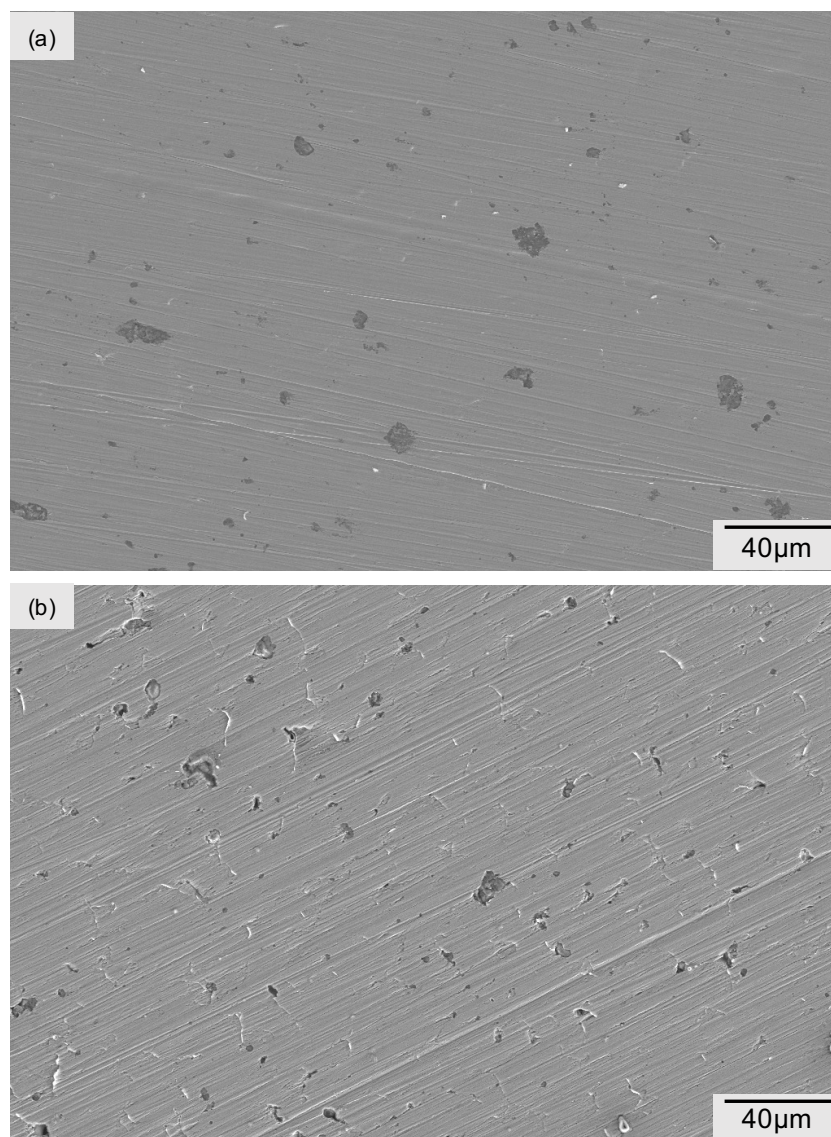


Figure 18 – Low magnification imaging of an FeMn coupon, showing a similar porous structure both before (a) and after (b) the acid etching procedure. The acid treatment has cleared the pores of Mn oxide.

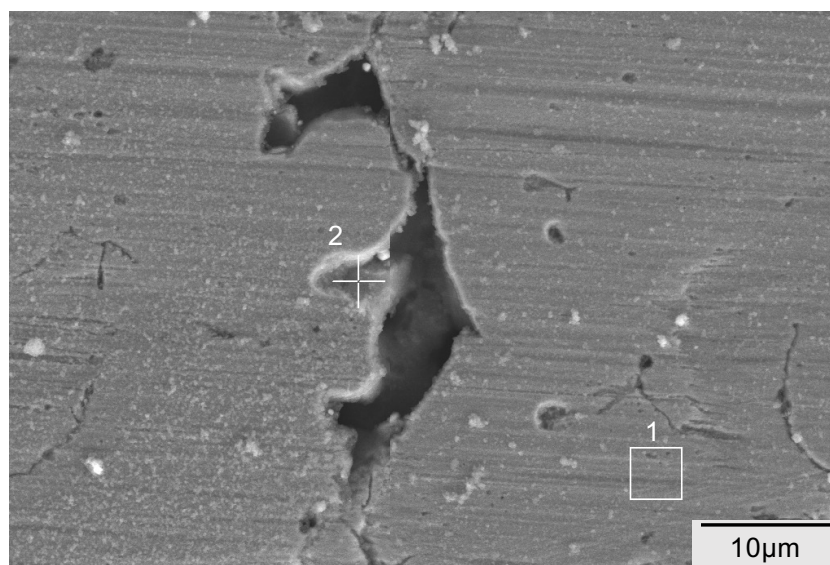


Figure 19 – High magnification imaging of an FeMn coupon . The acid etching procedure has removed Mn oxide within its pores. EDS analyses of the coupon’s surface (1) and pores (2) were performed.

Table 21 – EDS analyses of the surface (1) and pores (2) of an FeMn coupon after the acid etching procedure, showing the weight percentages of the detected elements.

Element	Fe	Mn	O	C	N	Cl
%Wt – 1	54.90	30.06	5.09	5.18	3.78	0.99
%Wt – 2	56.00	30.44	5.86	4.02	2.88	0.81

4.1.2.3. Iron-Manganese-Silver

The acid etching procedure had no effect on the structure of either FeMn1Ag or FeMn5Ag, as shown in Figure 20. Ag was still seen to be dispersed as discrete clumps within the coupon but Mn oxide was cleared from the pores of both coupons. As was seen before acid etching, the Fe to Mn ratio remained 65:35, this being the case both within the pores and on the surfaces of the coupons from both materials, as seen in Table 22. The O content on both

coupons' surfaces also remained unchanged, meaning that no new oxidation had occurred during the acid etching process.

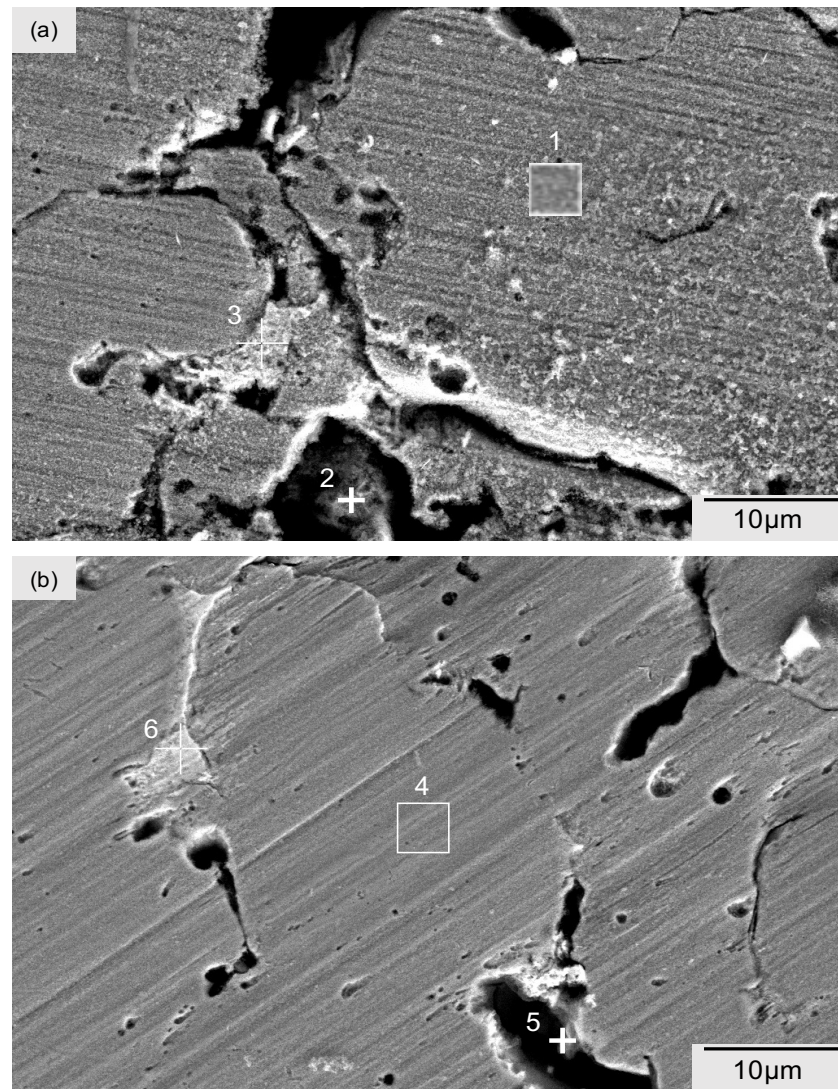


Figure 20 – SEM imaging of FeMn1Ag (a) and FeMn5Ag (b) coupons, showing a structure which remained unchanged by acid treatment. The acid etching procedure cleared the pores of Mn oxide. EDS analyses of the coupons' surfaces (1, 4), pores (2, 5) and bright clumps (3, 6) were also performed.

Table 22 – EDS analyses of the surfaces (1, 4), pores (2, 5) and bright areas (3, 6) of FeMn1Ag (1-3) and FeMn5Ag (4-6) coupons, showing the weight percentages of the detected elements.

Element	Fe	Mn	Ag	O	C	N	Cl	Si	Mg
%Wt – 1	52.71	29.98	-	7.25	6.07	3.47	0.33	0.19	-
%Wt – 2	52.88	29.49	-	6.91	5.50	3.20	2.02	-	-
%Wt – 3	4.97	9.42	53.63	21.50	7.80	1.99	0.34	-	0.35
%Wt – 4	59.55	28.24	-	2.37	5.78	3.80	-	0.25	-
%Wt – 5	51.83	26.08	-	1.94	16.41	3.73	-	-	-
%Wt – 6	4.71	5.61	73.04	6.31	9.84	-	-	-	0.48

4.1.3. X-Ray Diffraction Analysis of Iron Alloy Coupons and Pins

XRD examination of acid treated Fe alloy coupons and pins was performed in order to ascertain which phase of FeMn was present. As can be seen in Figure 21, the austenitic phase is present within all of the Mn-containing coupons, irrespective of whether they contain Ag or not. This was the intended outcome of the sintering process, γ -FeMn being confirmed to be stable at room temperature in these alloys. This also means that these materials should be compatible with MRI. Pure Fe on the other hand does not have an austenitic phase which is stable at room temperature and is only found to be present in its ferrite (α -Fe) form.

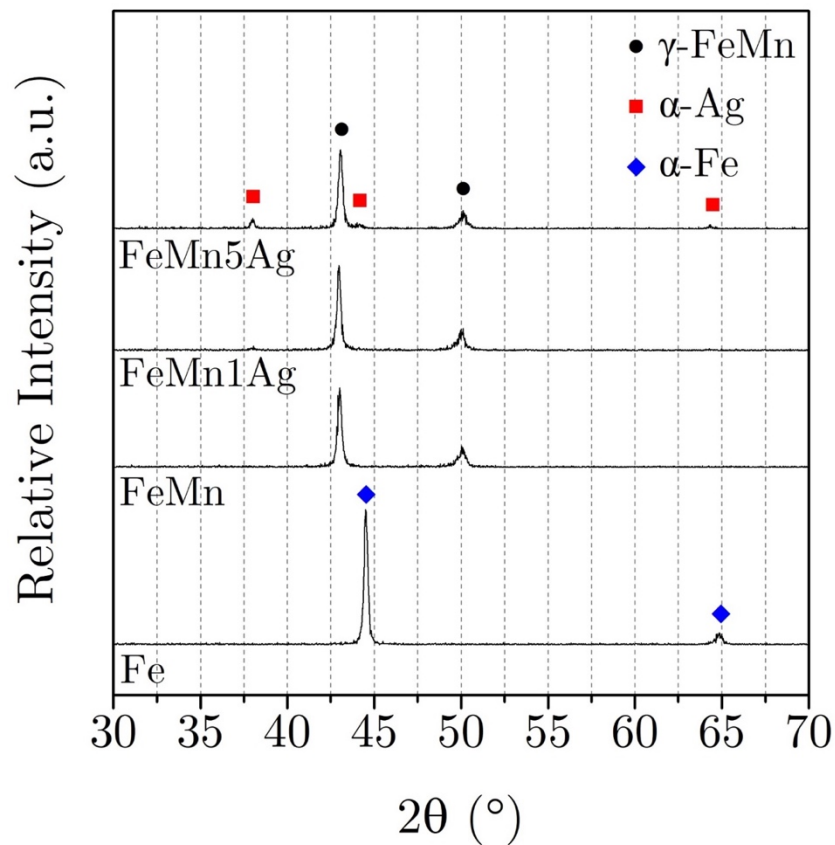


Figure 21 – XRD analysis of the test Fe alloy coupons and pins before implantation. The austenitic phase of FeMn is present in Mn-containing coupons, with Ag peaks also being present in FeMn1Ag and FeMn5Ag coupons. Pure Fe is present in its ferrite phase.

4.2. *In Vitro* Cytotoxicity

Considering the small sample sizes used during the *in vitro* cytotoxicity tests, testing for normality was difficult. For this reason, the parametric ANOVA test was employed.

4.2.1. As-Ground Coupons using a Human Foetal Osteoblast Cell Line

This set of tests involved the first run of *in vitro* cytotoxicity tests performed as part of this dissertation. All the materials tested here, including all the test Fe alloy and SS316LVM

coupons, were not corroded prior to the start of the test and were sterilised immediately before their use, limiting exposure time to moisture and resultant corrosion.

In the first instance, a two-way ANOVA model with interaction analysis was performed with luminescence reading as the dependent variable, as shown in Table 23. This analysis revealed that cell viability (given as luminescence) varied significantly between the materials tested, and also over the length of the test (this referring to whether cell viability testing was performed after one, two or four days of exposure to the materials in question). In both cases, $P < 0.001$. In addition to this, the interaction between material and length of test was also found to be significant, with $P < 0.001$.

Table 23 - Two-way ANOVA model with interaction analysis showing the effects of the material tested and length of testing (one, two and four days of exposure to the test materials) on hFOB cell viability.

Two-way ANOVA Model with Interaction – hFOB Cytotoxicity					
Dependent Variable: Luminescence					
Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P-value
Corrected Model	23642376 3825	20	11821188 191.258	35.952	<.001
Intercept	54342978 592	1	54342978 592.073	165.275	<.001
Material	15168272 1097	6	25280453 516.268	76.886	<.001
Day	22193934 776	2	11096967 388.117	33.750	<.001
Material * Day	61809719 971	12	51508099 97.602	15.665	<.001
Error	10850503 378	33	32880313 2.672		
Total	30143376 7620	54			
Corrected Total	24727426 7203	53			
R Squared = 0.956					

Considering this result, it was then necessary to run further analyses in order to determine which of the tested materials were responsible for significant variations in cell viability. To this end, further ANOVA tests were run on individual materials, this analysis showing that FeMn1Ag, FeMn5Ag, SS316LVM and Thermanox™ were the likely cause of this. These results are shown in Table 24.

Table 24 – One-way ANOVA model showing changes in hFOB cell viability over the four days of cytotoxicity testing, for each of the materials tested.

One-way ANOVA Model – hFOB Cell Viability for each Material				
Material	Day	Mean	Std. Deviation	P-value
Medium (Negative Control)	1	103.00	.	
	2	84.00	.	
	4	105.00	.	
Fe	1	542.33	664.511	0.312
	2	113.67	53.107	
	4	58.33	8.021	
Fe-Mn	1	121.00	68.505	0.359
	2	82.33	6.351	
	4	71.00	18.330	
Fe-Mn-1Ag	1	107.67	20.841	0.007
	2	71.33	5.508	
	4	58.67	1.528	
Fe-Mn-5Ag	1	126.00	10.817	<0.001
	2	81.00	9.539	
	4	60.33	2.082	
Thermanox™ (Positive Control)	1	41764.33	21318.638	0.005
	2	47657.67	10278.563	
	4	182504.00	58078.600	
SS316LVM (Positive Control)	1	81972.50	2727.311	0.015
	2	89857.50	5722.615	
	4	272969.50	54248.525	

Both of the positive controls SS316LVM ($P=0.015$) and Thermanox™ ($P=0.005$) showed good cell viability, with rising luminescence signals being recorded for both of these materials over the four days of testing. This result evidenced a multiplying population of hFOB cells in direct contact with these materials.

Results were different for the four test Fe alloy materials however, with the Ag-containing alloys showing decreasing luminescence readings over the four-day test period. Significance values of $P=0.007$ for FeMn1Ag and $P<0.001$ for FeMn5Ag were obtained. The absolute values for luminescence obtained for both of these materials were always similar to those obtained for the medium only negative control which never contained any cells, suggesting that there were never any viable cells growing in direct contact with these coupons.

Both pure Fe and FeMn showed no significant change in cell viability over the four-day cytotoxicity test, with luminescence values being similar to the medium only negative control ($P>0.05$). Of note is that pure Fe showed an initial cell viability, with luminescence readings indicating the possible presence of some cells on these coupons' surfaces after one day of exposure to this material. In spite of this however, standard deviation was noted to be high for these values, possibly indicating an erroneous reading. Luminescence readings fell by the second day and were then similar to those seen for the medium only negative control. A visual representation of the luminescence results is plotted in the form of a bar chart, as seen in Figure 22.

hFOB Cell Viability

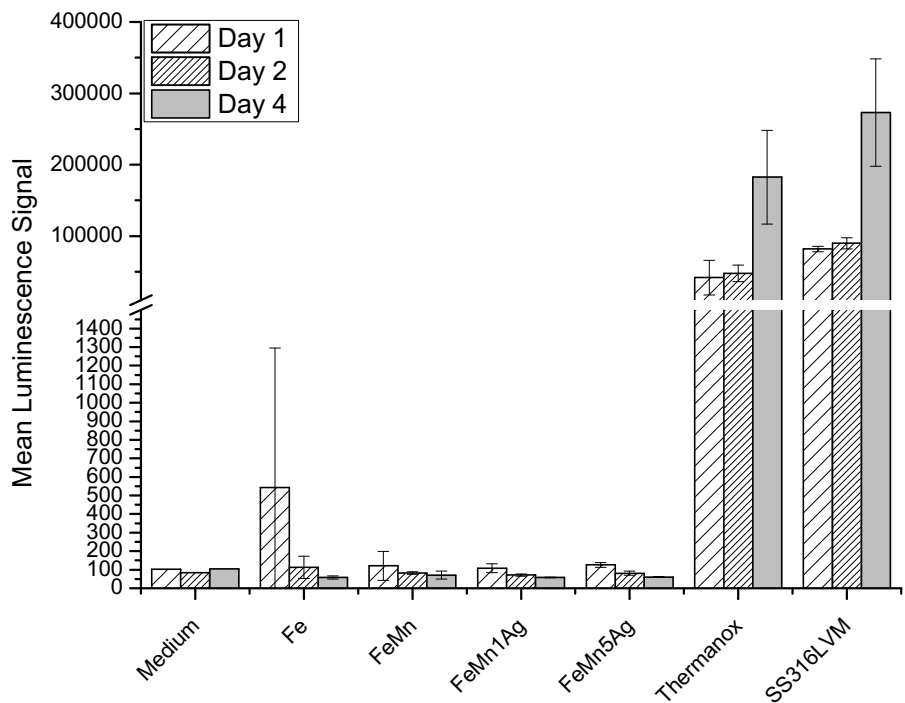


Figure 22 – Bar chart depicting the cell viability of hFOB cells seeded onto test Fe alloy materials. The height of each bar represents the mean luminescence signal obtained for three technical replicates of each Fe alloy material on each day of testing. Negative control (medium with no cells) and positive control (three Thermanox™ coverslips and three SS316LVM coupons) comparators are also plotted. Error bars indicate the standard error multiplied by 1.96, giving the confidence interval. Luminescence readings for test Fe alloy materials are similar to the negative control (medium with no cells), indicating significant cytotoxicity.

A separate ANOVA test was then performed as seen in Table 25. This showed how cell viability varied between materials on each of the days in which viability tests were run, be it after one, two or four days of exposure to the tested materials. This analysis was also significant with differences between materials being noted for every day of testing ($P < 0.001$ in every case). Considering the results shown in Figure 22 and Table 24, it must have been the positive controls SS316LVM and Thermanox™ which accounted for this difference.

Table 25 – One-way ANOVA model showing differences in hFOB cell viability between materials, after one, two and four days of exposure.

One-way ANOVA – Comparison of Materials' hFOB Cell Viability on each Day				
Day	Material	Mean	Std. Deviation	P-value
1	Medium	103.00	.	<0.001
	Fe	542.33	664.511	
	Fe-Mn	121.00	68.505	
	Fe-Mn-1Ag	107.67	20.841	
	Fe-Mn-5Ag	126.00	10.817	
	Thermanox™	41764.33	21318.638	
	SS316LVM	81972.50	2727.311	
2	Medium	84.00	.	<0.001
	Fe	113.67	53.107	
	Fe-Mn	82.33	6.351	
	Fe-Mn-1Ag	71.33	5.508	
	Fe-Mn-5Ag	81.00	9.539	
	Thermanox™	47657.67	10278.563	
	SS316LVM	89857.50	5722.615	
4	Medium	105.00	.	<0.001
	Fe	58.33	8.021	
	Fe-Mn	71.00	18.330	
	Fe-Mn-1Ag	58.67	1.528	
	Fe-Mn-5Ag	60.33	2.082	
	Thermanox™	182504.00	58078.600	
	SS316LVM	272969.50	54248.525	

4.2.2. Pre-Corroded Coupons using an MC3T3-E1 Cell Line

Considering the results obtained from the cytotoxicity tests involving the hFOB cell line, it was decided to perform a second biological replicate using MC3T3-E1 cells. In this case however, test Fe alloy coupons would be pre-corroded for a period of three days prior to being seeded with cells, as corrosion rate (and resultant cytotoxicity) would be expected to decrease over time, resulting in improved cell viability. In order to test this, a further coupon of each test Fe alloy material was seeded in its as-ground, uncorroded state, and the results analysed

for significance. This was the first analysis performed and showed no difference in cell viability between three-day pre-corroded and uncorroded test Fe alloy coupons ($P=0.999$). In view of this result, corrosion status was removed as a variable and all subsequent analyses involving the MC3T3-E1 cell line were performed taking only luminescence signals obtained from pre-corroded coupons into account.

A two-way ANOVA model with interaction analysis was then performed, as shown in Table 26, with luminescence reading as the dependent variable. Similar to the results obtained in the hFOB cytotoxicity tests, cell viability was seen to vary significantly between the materials tested, and also over the length of exposure to the materials in question with $P<0.001$ in both cases. In addition, the interaction between these two variables (material and length of exposure to test materials) was also found to be significant, with $P<0.001$.

As was done for the hFOB cell line, materials were then analysed individually in order to ascertain whether cell viability varied significantly with increasing length of exposure, as seen in Table 27. This revealed significant results for SS316LVM ($P<0.001$) and FeMn5Ag ($P=0.039$). All other materials failed to show significant variations in cell viability over the same four-day test period ($P>0.05$).

Table 26 - Two-way ANOVA model with interaction analysis showing the effects of the material tested and length of testing (one, two and four days of exposure to the test materials) on MC3T3-E1 cell viability.

Two-way ANOVA model with interaction – MC3T3-E1 Cytotoxicity					
Dependent Variable: Luminescence					
Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	P-value
Corrected Model	22748013 33371.166	20	11374006 6668.558	23.856	<.001
Intercept	38603604 7818.292	1	38603604 7818.292	80.969	<.001
Material	12147163 10714.830	6	20245271 8452.472	42.463	<.001
Day	25468490 0213.759	2	12734245 0106.880	26.709	<.001
Material * Day	83347745 2236.214	12	69456454 353.018	14.568	<.001
Error	15733397 3288.333	33	47676961 60.253		
Total	27827458 16793.000	54			
Corrected Total	24321353 06659.500	53			
R Squared = 0.935					

As in the hFOB tests, SS316LVM exhibited good cell viability with an increasing luminescence throughout the test. Despite also showing increasing luminescence, Thermanox™ failed to show statistically significant differences in cell viability over the same four-day period ($P=0.127$). This was likely due to a large inter-sample variability, as seen from the large standard deviation in Table 27.

Interestingly, FeMn5Ag was found to have a significantly increased luminescence signal ($P=0.039$) after four days of exposure of cells to this material, suggesting the possibility of cells growing in direct contact with this material. Despite this, absolute values for luminescence were very similar to that obtained for the medium only negative control, suggesting that this

reading was still not significant for any cell viability. A visual representation of the obtained results is plotted in the form of a bar chart, as seen in Figure 23.

Table 27 - One-way ANOVA model showing changes in MC3T3-E1 cell viability over the four days of cytotoxicity testing, for each of the materials tested.

One-way ANOVA Model – MC3T3-E1 Cell Viability for each Material				
Material	Day	Mean	Std. Deviation	<i>P</i> -value
Medium (Negative Control)	1	56.00	.	
	2	64.00	.	
	4	124.00	.	
Fe	1	102.33	63.799	0.455
	2	62.00	12.767	
	4	69.67	18.771	
Fe-Mn	1	79.00	17.692	0.374
	2	61.00	20.298	
	4	58.00	16.523	
Fe-Mn-1Ag	1	79.67	9.018	0.109
	2	73.67	15.695	
	4	58.00	4.000	
Fe-Mn-5Ag	1	67.67	1.528	0.039
	2	54.33	7.767	
	4	92.67	22.811	
Thermanox™ (Positive Control)	1	40168.00	25517.844	0.127
	2	132023.67	40164.892	
	4	348336.00	271618.637	
SS316LVM (Positive Control)	1	82433.00	15185.825	<0.001
	2	311958.50	70721.285	
	4	999009.50	4441.338	

MC3T3-E1 Cell Viability

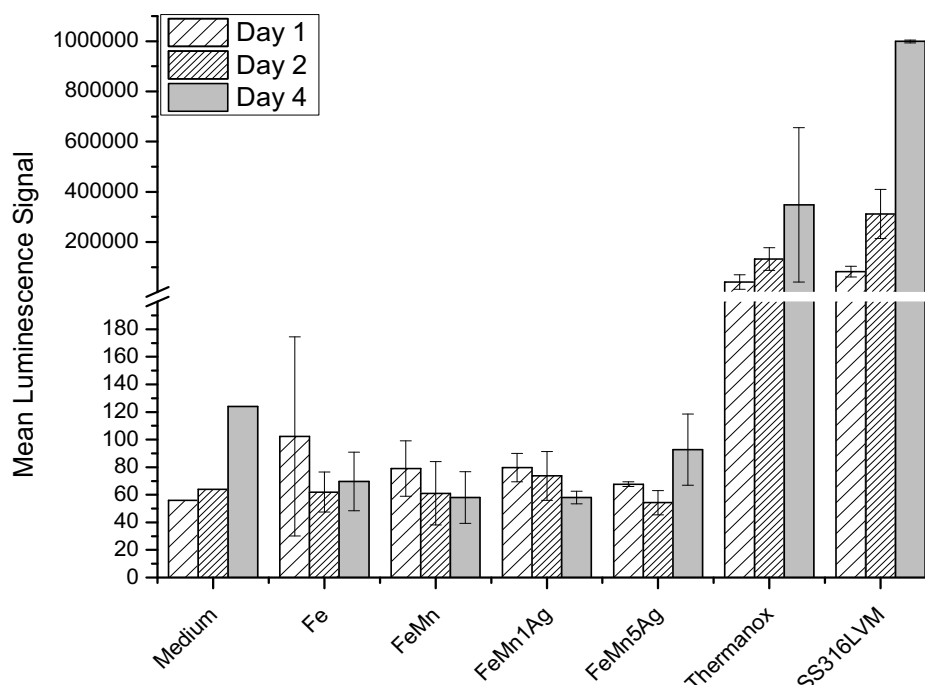


Figure 23 – Bar chart depicting the cell viability of MC3T3-E1 cells seeded onto test Fe alloy materials. The height of each bar represents the mean luminescence signal obtained for three technical replicates of each Fe alloy material on each day of testing. Negative control (medium with no cells) and positive control (three Thermanox™ coverslips and three SS316LVM coupons) comparators are also plotted. Error bars indicate the standard error multiplied by 1.96, giving the confidence interval. Luminescence readings for test Fe alloy materials are similar to the negative control (medium with no cells), indicating significant cytotoxicity.

Further analysis was performed using a one-way ANOVA test, as seen in Table 28. This test was run in order to identify any significant differences in cell viability occurring between materials, on each day the CellTiter-Glo® cell viability test was run. This analysis showed significant differences at each of one, two and even four days of exposure to the test materials, including positive controls. As was the case for the hFOB cytotoxicity tests, these differences were due to the Thermanox™ and SS316LVM positive controls, as both of these materials had cells growing on their surfaces while no cells were found to be present on any of the other test Fe alloy materials.

Table 28 - One-way ANOVA model showing differences in MC3T3-E1 cell viability between materials, after one, two and four days of exposure.

One-way ANOVA – Comparison of Materials' MC3T3-E1 Cell Viability on each Day				
Day	Material	Mean	Std. Deviation	P-value
1	Medium	56.00	.	<0.001
	Fe	102.33	63.799	
	Fe-Mn	79.00	17.692	
	Fe-Mn-1Ag	79.67	9.018	
	Fe-Mn-5Ag	67.67	1.528	
	Thermanox™	40168.00	25517.844	
	SS316LVM	82433.00	15185.825	
2	Medium	64.00	.	<0.001
	Fe	62.00	12.767	
	Fe-Mn	61.00	20.298	
	Fe-Mn-1Ag	73.67	15.695	
	Fe-Mn-5Ag	54.33	7.767	
	Thermanox™	132023.67	40164.892	
	SS316LVM	311958.50	70721.285	
4	Medium	124.00	.	<0.001
	Fe	69.67	18.771	
	Fe-Mn	58.00	16.523	
	Fe-Mn-1Ag	58.00	4.000	
	Fe-Mn-5Ag	92.67	22.811	
	Thermanox™	348336.00	271618.637	
	SS316LVM	999009.50	4441.338	

4.3. Imaging of the Pre-Corroded Coupons used for *In Vitro* Cytotoxicity Testing

Immediately after the conclusion of *in vitro* cytotoxicity testing using the MC3T3-E1 cell line, the pre-corroded Fe alloy and SS316LVM coupons were exposed to a cell fixing protocol, as in *Section 3.3.3.1 – Cell Fixing Protocol*. Gold sputter-coating of these same coupons was then performed with subsequent examination under reflective light microscopy and SEM

imaging. This allowed the visualisation of cells present on the SS316LVM positive control, as well as the absence thereof on the test Fe alloy coupons.

Reflective light microscopy was performed in favour of transmitted light microscopy because of the coupons' opacity. This prevents the passage of light emitted in a top-down microscope from reaching the objective lens, precluding examination using this method. Reflective light microscopy images, as seen in Figure 24, confirmed the absence of cell growth on the surfaces of any of the test Fe alloy coupons, on any of the days of testing. Instead, the accumulation of corrosion products on the surfaces of all of these coupons was clearly seen, with FeMn in particular showing areas of seemingly increased corrosion product formation. Figure 24(b) shows the reflective light microscopy images obtained for FeMn, particularly an area containing the boundary between relatively more and less corroded regions.

In contrast, SS316LVM showed cells growing on the surfaces of the coupons on all of the four days of testing, with no sign of accumulation of corrosion product. Examination of the day one coupon revealed cells interspersed on its surface, but these had replicated sufficiently by the fourth day of testing to become completely confluent, as seen in Figure 24(f).

SEM imaging is seen in Figure 25 and evidences the absence of any cellular attachment or growth on any of the test Fe alloy materials throughout the four-day *in vitro* cytotoxicity test. The accumulation of corrosion products on the surfaces of these coupons is also seen, Figure 25(b) in particular showing an area on the FeMn coupon's surface with a boundary between relatively more and less corroded regions. In contrast, SEM images of SS316LVM show the absence of accumulation of corrosion products, instead evidencing a surface which remains unchanged throughout the testing period. In addition, cell number and concentration are seen to increase, with complete confluence of cells occurring by the fourth day of testing, as seen in Figure 25(f).

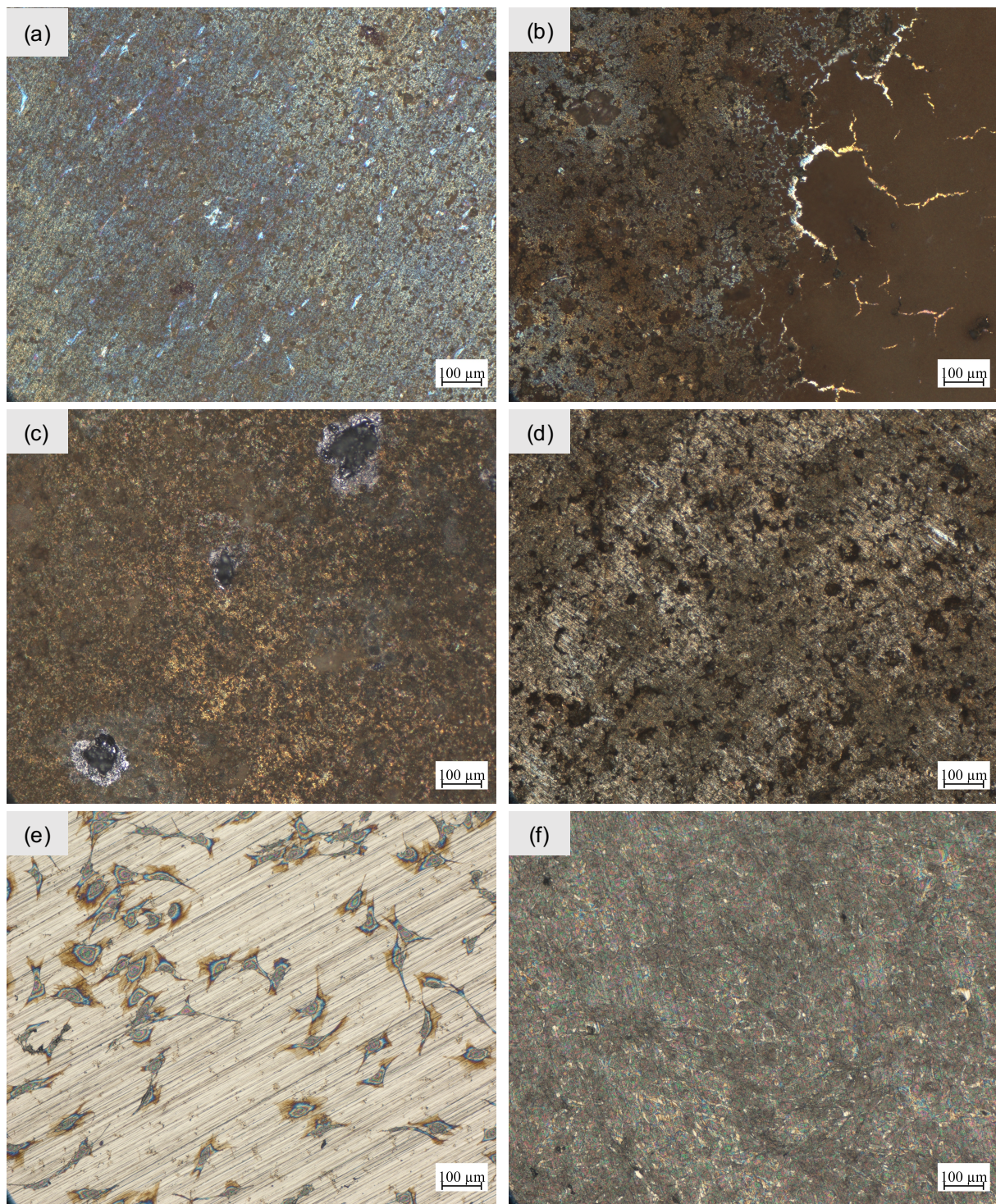


Figure 24 – Reflective light microscopy images of pure Fe (a), FeMn (b), FeMn1Ag (c) and FeMn5Ag (d) coupons after four days of *in vitro* cytotoxicity testing. Also imaged are SS316LVM coupons after one (e) and four (f) days of testing.

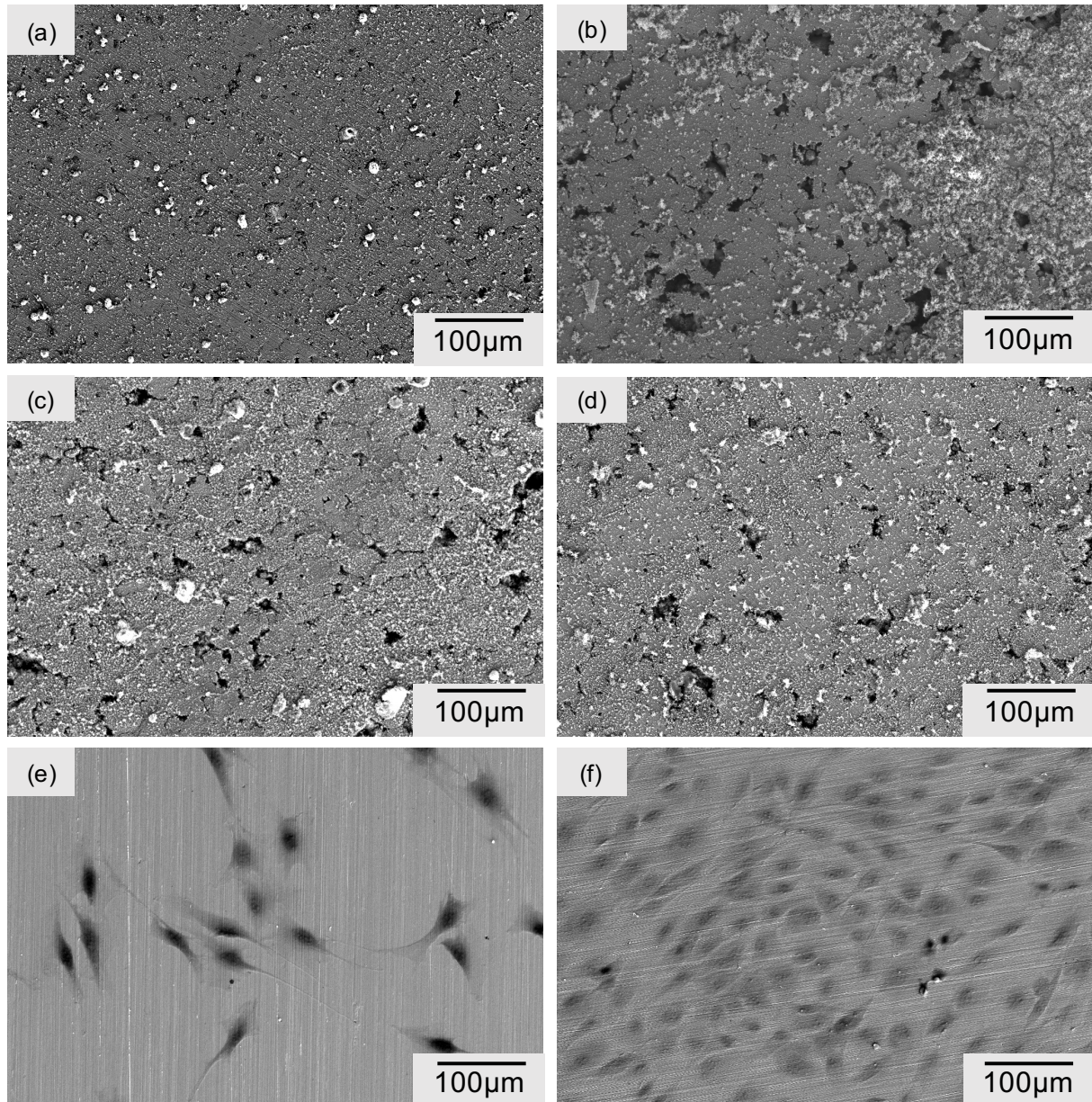


Figure 25 – SEM images of pure Fe (a), FeMn (b), FeMn1Ag (c) and FeMn5Ag (d) coupons after four days of *in vitro* cytotoxicity testing. Also imaged are SS316LVM coupons after one (e) and four (f) days of testing.

4.4. Metallographic Analysis of Test Fe Alloy and Stainless Steel 316LVM Coupons After *In Vitro* Cytotoxicity Tests

As SEM imaging of the pre-corroded test Fe alloy and SS316LVM coupons was being performed, characterisation using EDS was performed simultaneously. As there was no cell

growth noted throughout the four days of *in vitro* cytotoxicity testing on any of the test Fe alloy coupons, EDS was only performed on those coupons that had viability testing performed after the fourth day. This was done as it was thought that corrosion rates would vary between the four test Fe alloy coupons, with differences becoming most evident by the fourth day of testing.

4.4.1. Pure Iron

SEM imaging of the pure Fe coupons, as seen in Figure 26, revealed that the majority of their surfaces were uniformly corroded. There were however, areas of seemingly increased corrosion, as evidenced in Figure 26(b). These areas were small in size and few in number, representing a minority of the overall surfaces of the pure Fe coupons. They were much rougher in appearance than the remainder of the uniformly corroded surface.

EDS analyses of both regions were performed, confirming the presence of corrosion product in both cases, as seen in Table 29. The amount of O present in the area represented in Figure 26(b) however confirms that these areas contained relatively greater amounts of corrosion product.

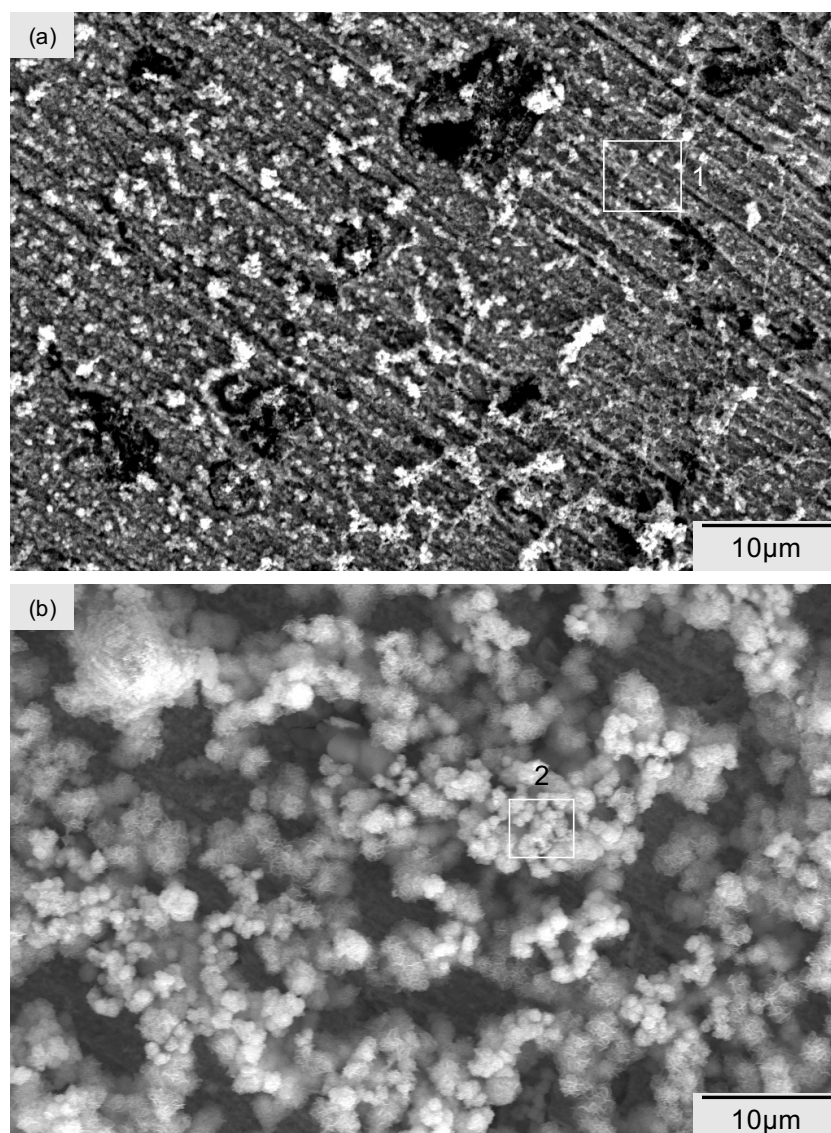


Figure 26 – SEM imaging of a pure Fe coupon’s surface after four days of *in vitro* cytotoxicity testing. The majority of the coupon’s surface was uniformly corroded (a) but there were also areas of apparently increase corrosion (b).

EDS analyses were performed of both the uniformly corroded (1) and increased corrosion (2) areas.

Table 29 – EDS analyses performed on areas of uniform (1), and seemingly increased (2) corrosion on the surface of a pure Fe coupon after four days of *in vitro* cytotoxicity testing. Weight percentages of the detected elements are shown.

Element	Fe	O	C	N	Na	Mg	Al	Si	P	Ca
%Wt – 1	85.97	5.94	5.43	2.02	0.64	-	-	-	-	-
%Wt – 2	49.18	28.93	11.94	4.10	2.50	0.46	0.11	0.17	2.21	0.40

4.4.2. Iron-Manganese

SEM imaging of the surface of the FeMn coupon, as shown in Figure 27, confirmed that it had corroded uniformly over the majority of its surface. This material was noted to have larger amounts of, and more frequent corrosion products scattered on its surface than was seen for pure Fe (Refer to Figure 26(a)). EDS analysis, as shown in Table 30, confirms the greater O content in these areas than over the general remaining surface of these coupons.

As was also seen with the pure Fe coupon, the FeMn coupon evidenced areas of seemingly increased corrosion as compared to the remainder of its surface, as seen in Figure 27. These were larger and more frequent than was seen with pure Fe, however they still represented a minority of the surface. They were confirmed to be composed of a large amount of O, very similar to that evidenced by the corrosion products on the remainder of its surface, confirming them to be areas of increased corrosion. These areas were also viewed under reflective light microscopy (Refer to Figure 24(b)).

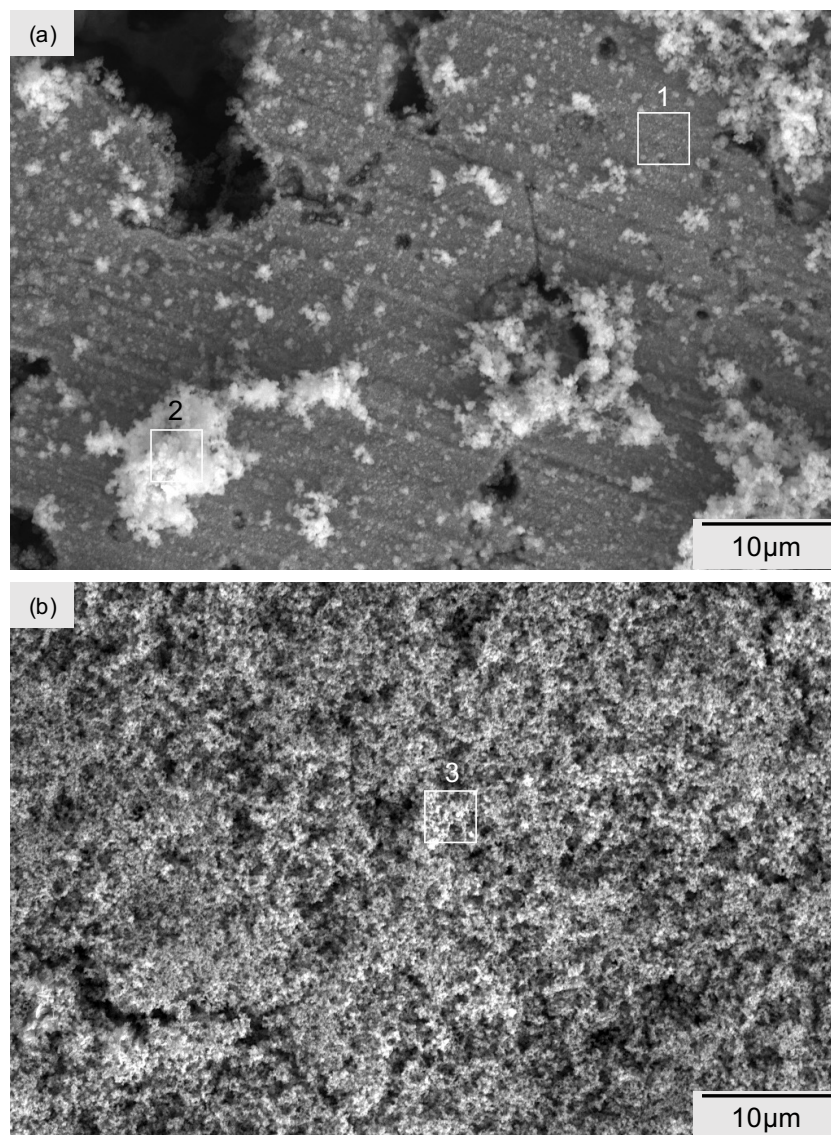


Figure 27 – SEM imaging of an FeMn coupons' surface after four days of *in vitro* cytotoxicity testing. The majority of the coupon's surface was uniformly corroded (a) but there were also areas of apparently increase corrosion (b). EDS analyses of the uniformly corroded (1) surface, as well as of corrosion products (2) and also of areas of increased corrosion (3) were performed.

Table 30 – EDS analyses performed on areas of uniform (1) corrosion as well as corrosion products (2) present over the majority of the surface of an FeMn coupon. Areas of seemingly increased (3) corrosion have also been analysed with weight percentages of the detected elements being shown.

Element	Fe	Mn	O	C	N	Na	Al	Si	P	Ca
%Wt – 1	61.60	22.98	6.03	5.62	2.17	0.43	0.19	0.19	0.59	0.19
%Wt – 2	43.37	10.77	26.04	9.18	3.02	2.37	0.08	0.27	4.03	0.85
%Wt – 3	46.14	5.85	28.31	9.42	3.21	2.58	0.08	0.47	3.09	0.86

4.4.3. Iron-Manganese-Silver

FeMn1Ag and FeMn5Ag had similar appearances in that both were seen to have uniformly corroded surfaces, as seen in Figure 28. There was no evidence of the bare, uncorroded metal still present in either of these materials, nor was there evidence of areas of seemingly increased corrosion, as was seen in pure Fe (Figure 26(b)) and FeMn (Figure 27(b)). EDS analysis, as seen in Table 31, confirms the presence of large amounts of O in both cases. It was present in greater amounts in the case of FeMn5Ag however, confirming the formation of greater amounts of corrosion product.

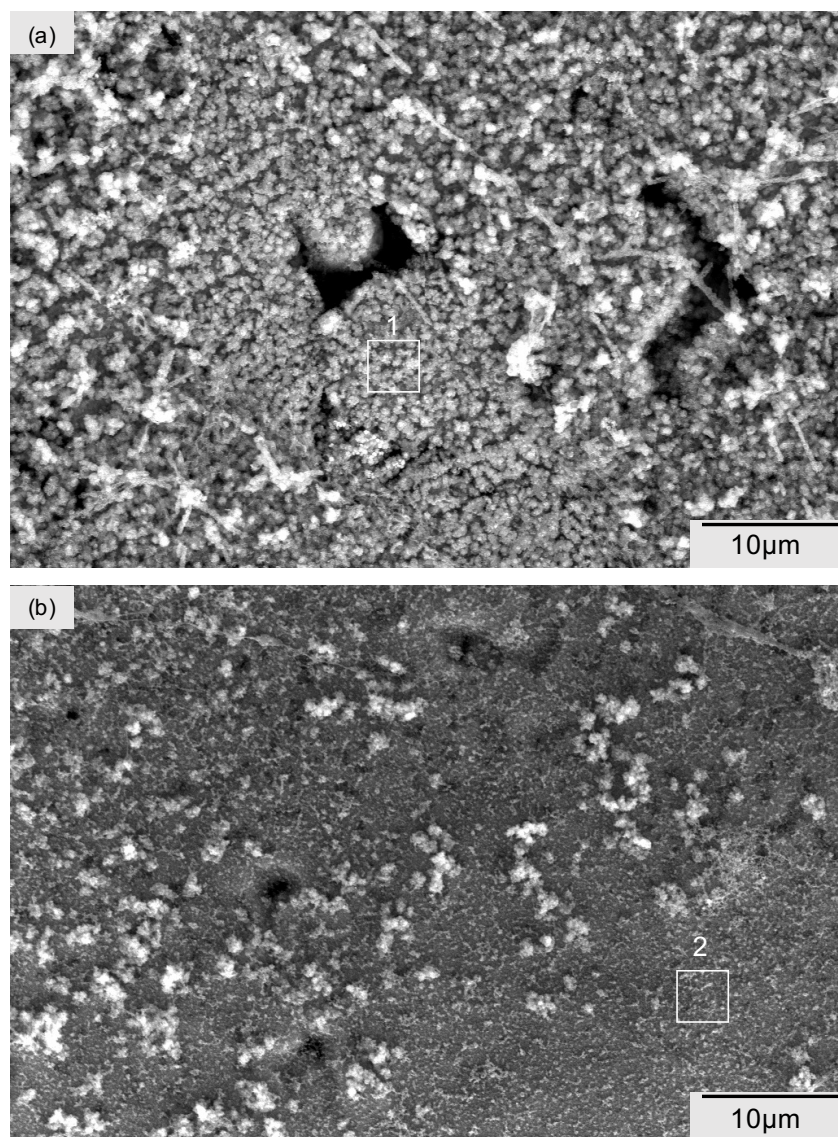


Figure 28 – SEM imaging of FeMn1Ag (a) and FeMn5Ag (b) coupons after four days of *in vitro* cytotoxicity testing. EDS analyses of the surfaces of both FeMn1Ag (1) and FeMn5Ag (2) was performed.

Table 31 – EDS analyses of the surfaces of FeMn1Ag (1) and FeMn5Ag (2) coupons showing the weight percentages of the detected elements.

Element	Fe	Mn	Ag	O	C	N	Na	Mg	Si	P	Ca	Al
%Wt – 1	48.00	27.02	0.23	11.74	8.29	2.40	1.06	0.21	0.21	0.47	0.37	-
%Wt – 2	52.02	3.64	0.00	29.72	9.45	2.67	0.79	0.17	0.30	0.81	0.35	0.07

4.4.4. Stainless Steel 316LVM

SEM imaging of SS316LVM is shown in Figure 29 and evidences the lack of formation of corrosion products on its surface. There are clearly evident grinding marks, these having been introduced during the preparation stages of the coupon. These are similar to the pre-testing marks and have remained prominent and uncorroded.

The presence of MC3T3-E1 cells on the surface of the SS316LVM coupons is also clearly visible. The cell seen in Figure 29 has a normal morphology, is adherent to the SS316LVM coupon surface and is growing adjacent to other similar cells.

EDS analyses were performed of both cellular and acellular regions of the SS316LVM coupon, as shown in Table 32. The acellular regions, containing no cellular material have no O present, evidencing the lack of formation of corrosion products. Instead, the elements Ni (Nickel), Mo (Molybdenum) and Cr were detected, these being integral parts of SS316LVM. Analysis of a cellular section shows a very different material composition. The Fe content is markedly lower, along with Ni, Cr and Mo to a lesser, but still appreciable extent, as the surface analysis now takes biological material into account. The C, N and O content along with Na, Cl and P, are all increased as these are very prominent intra-cellular elements.

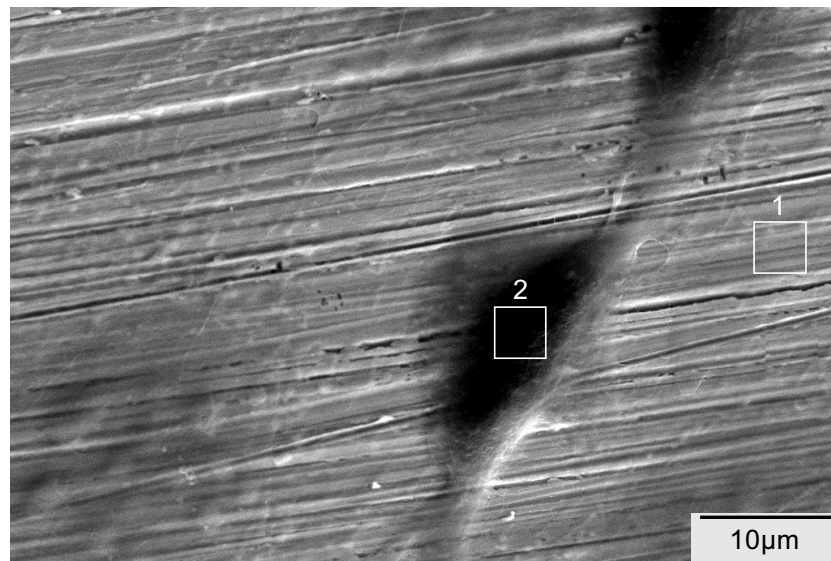


Figure 29 – SEM image of an SS316LVM coupon after four days of *in vitro* cytotoxicity testing. MC3T3-E1 cells are easily appreciable on the surface. EDS analyses of both acellular (1) and cellular (2) region were performed.

Table 32 – EDS analyses of the acellular (1) and cellular (2) regions of an SS316LVM coupon showing the weight percentages of the detected elements.

Element	Fe	Mn	Ni	Cr	Mo	O	C	N	Na	Cl	P
%Wt – 1	52.30	1.60	12.49	15.34	2.72	0.00	11.24	3.20	-	0.10	0.18
%Wt – 2	24.69	0.81	5.11	7.97	2.39	6.43	41.74	8.16	0.34	0.79	0.98

4.5. *In Vivo* Biocompatibility

All twenty-four of the rats underwent successful operative implantation of test Fe alloy pins with all animals surviving the operative procedure and immediate peri-operative period. There were no cases of surgical site infection, with none of the rats requiring removal of their sutures, this having been performed by the rats themselves. In addition, all rats continued to grow normally and went on to reach the six-month post-operative mark, when euthanasia was performed. Throughout this six-month observation period, none of the rats exhibited any

signs of stress and hence none of the rats had to be euthanised prior to the planned end of *in vivo* experimentation.

By the end of the experiment, all of the retained pins were well fixed within bone. None of the pins were loose and those subjected to material characterisation had to be prised out of the bone within which they had been implanted.

4.5.1. Computed Tomography

After euthanasia of the rats was performed, the tails were collected and sent for CT imaging, as show in Figure 30. This produced an image which showed retention of the pins in thirteen of the twenty-four rats originally having implants sited. As the rats were separated from each other both before and after the surgical procedure, the only explanation for the disappearance of the pin was the removal of the implant by the rat itself in each case. Table 33 shows the number of pins lost and retained by material, FeMn being the most commonly lost pin.

In order to ensure that the pins were truly lost, each tail was inspected closely to ensure that the incisional scars were present on each of the specimens. The skin was subsequently removed and further examination was performed by breaking the relevant bones to ensure the physical absence of the pins. Some of the vertebrae with lost pins were not broken and were instead examined histologically.



Figure 30 – CT image of the twenty-four rat tails with numbers 1 and 24 labelled for reference. This image shows retention of the implanted pins (marked with arrows) in thirteen of the twenty-four rats.

Table 33 – Number of Fe alloy pins implanted, lost and retained by material.

Material	Number Implanted	Number Lost	Number Retained
FeMn	8	5	3
FeMn1Ag	8	2	6
FeMn5Ag	8	4	4

4.5.2. Magnetic Resonance Imaging

MR imaging was performed on rat bodies with tails known to have retained the implanted pins. Fe-sensitive sequences usually used to detect small amounts of Fe within the vital organs of patients known to have diseases such as Thalassaemia, were also employed. Despite close

examination of the MR images by a radiological expert, there were no appreciable signs of accumulation of Fe within any vital organs.

4.5.3. Histological Examination

Histological examination of demineralised bone from the implants' proximities revealed bony architectures which closely resembled that of normal bone. This was the case for all test Fe alloy materials. Control vertebrae obtained from the same rats but from sites other than the pin implantation site were also imaged. For comparison purposes, these histological images are seen together in Figure 31, which evidences similar bony architectures for both control vertebrae and vertebrae having had pins implanted.

The only visible difference was the presence of a dark discolouration of the decalcified matrix noted in those sections obtained from around an implanted pin, as seen in Figure 31(a). This was seen in all specimens, irrespective of whether they were obtained from around FeMn, FeMn1Ag or FeMn5Ag pins. A further example of this is seen in Figure 32. Importantly, examination of the vertebrae with the lost pins revealed an architecture which was exactly similar to control bones, and had no discolouration, as seen in Figure 33.

From gross examination of the pins, it was evident that very little corrosion had occurred. They maintained their cylindrical shape and had not decreased in size over the six-month implantation period. Closer examination of bone and soft tissues immediately surrounding the implant also did not show the presence of any grossly visible corrosion product. There was only a slight discolouration of bone in the immediate vicinity, corresponding to the darkened matrix noted on histological examination.

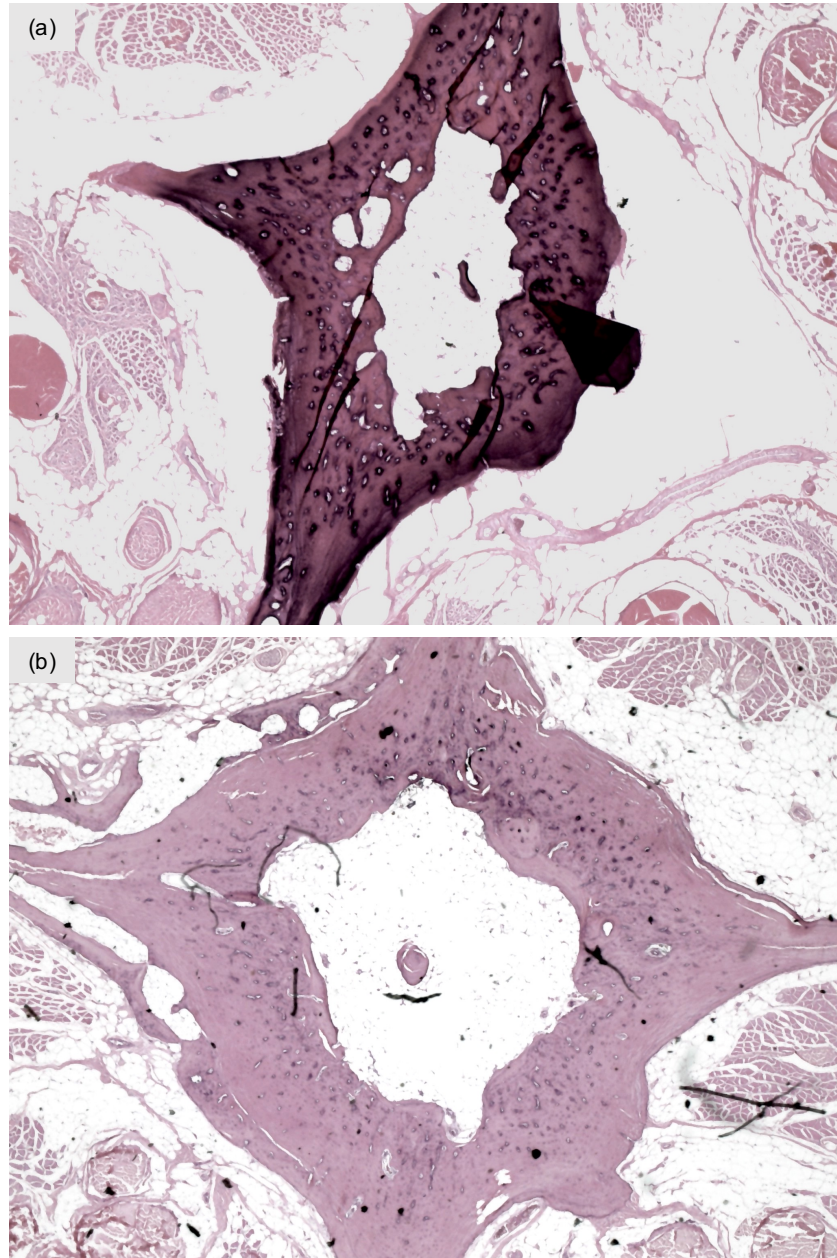


Figure 31 – Histological images obtained at x100 from (a) rat vertebra implanted with FeMn and (b) control vertebra.

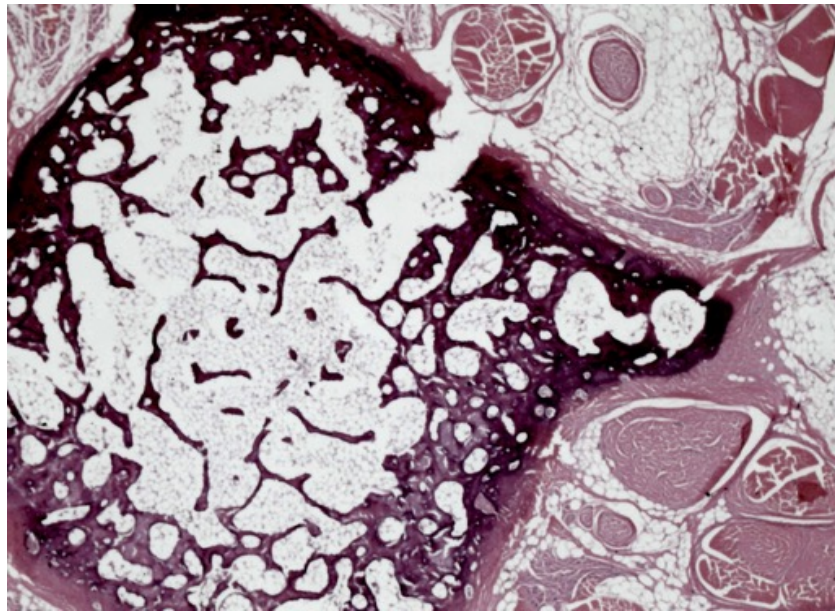


Figure 32 – Histological image obtained at x100 showing the darkening of decalcified matrix noted within specimens obtained from vertebrae implanted with Fe alloy pins.

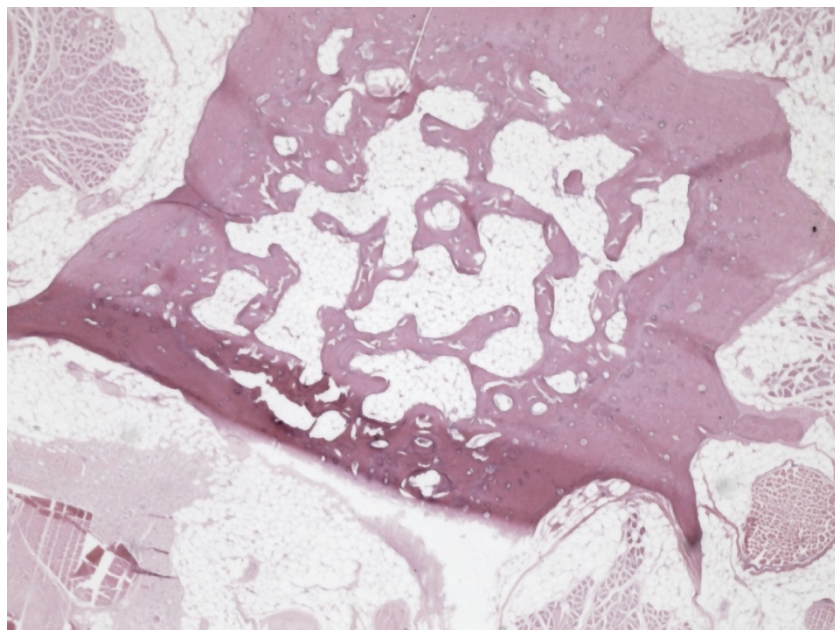


Figure 33 – Histological image at x100 showing normal bone architecture. This specimen was obtained from an area of bone where a lost pin had previously been implanted.

4.6. Metallographic Characterisation of the Explanted Pins

In order to understand the composition and amount of corrosion having formed on the different pins, different analyses were performed involving both their surfaces and their cut sections.

4.6.1. Characterisation of Explanted Pins' Surfaces

Macroscopic examination of all of the explanted pins revealed them to be uniformly black in colour with no areas of visibly exposed original Fe alloy material. Low magnification SEM imaging as seen in Figure 34, shows that the implants maintained their cylindrical shape throughout the six-month implantation period. The pins' surfaces remained uniform without evidence of the formation of cavities or the loss of significant amounts of material.

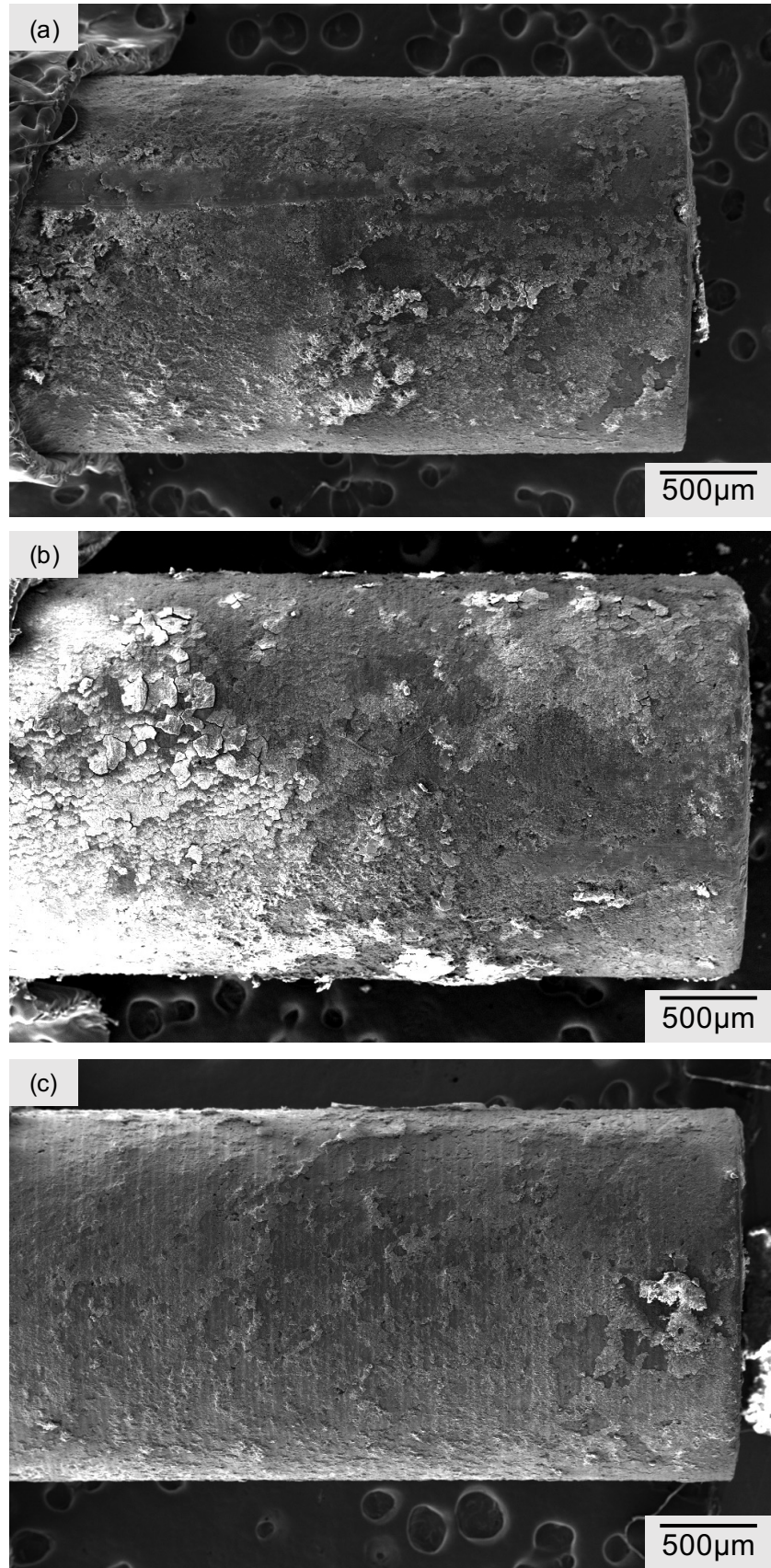


Figure 34 – Low magnification SEM images of the explanted pins showing (a) FeMn, (b) FeMn1Ag and (c) FeMn5Ag. All pins maintained their gross structure with very little corrosion visualised macroscopically.

Higher magnification SEM imaging of the explanted pins was also performed, as shown in Figure 35. Irrespective of whether the base material was FeMn, FeMn1Ag or FeMn5Ag, these images showed the development of two different layers on the pins' surfaces. The deeper layer was more uniform in appearance while the superficial layer had developed multiple cracks along its surface.

In order to identify these individual layers, EDS analyses of both were performed, as shown in Table 34. These analyses confirm the presence of two distinct layers, each with a different composition. The deeper layer (denoted by 2, 4 and 6 in Figure 35) contains relatively higher levels of Mn, with similar levels of O and Fe. In contrast, the more superficial layer (denoted by 1, 3 and 5 in Figure 35) contains greater amounts of Ca, P, Na and K, elements known to be present in cellular material and bone. No Ag was detected in any of the analyses performed.

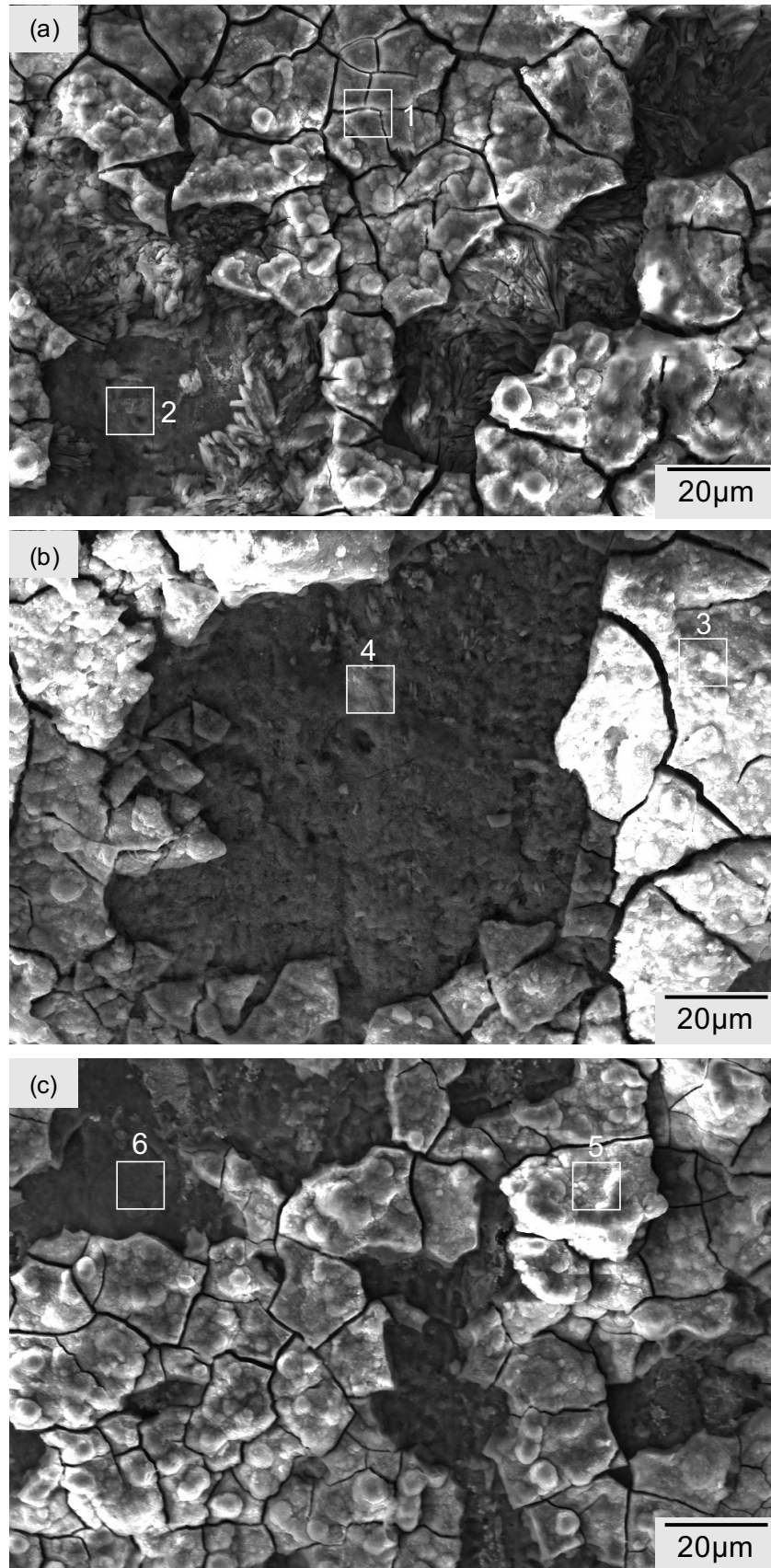


Figure 35 – High magnification SEM images of (a) FeMn, (b) FeMn1Ag and (c) FeMn5Ag pins showing the presence of two distinct layers on their surfaces. EDS analyses of the superficial (1, 3, 5) and deep (2, 4, 6) layers was performed.

Table 34 – EDS analyses of the explanted FeMn (1, 2), FeMn1Ag (3, 4) and FeMn5Ag (5, 6) pins with 1, 3 and 5 denoting the superficial layers and 2, 4 and 6 denoting the deeper layers. The weight percentages of the detected elements are shown.

Element	Fe	Mn	O	C	N	Ca	P	Na	S	K	Ag
%Wt – 1	19.89	2.17	42.37	17.12	3.89	3.60	8.48	1.12	0.31	1.04	-
%Wt – 2	18.22	8.05	35.65	28.08	6.10	0.56	1.35	0.42	1.57	-	-
%Wt – 3	12.79	2.45	43.30	25.01	6.64	1.67	5.40	1.16	0.35	0.89	-
%Wt – 4	10.01	5.46	38.50	35.73	5.50	1.31	2.25	0.33	0.44	0.11	-
%Wt – 5	21.16	1.53	40.15	18.60	4.23	3.10	7.90	1.09	0.57	1.47	-
%Wt – 6	20.50	9.53	44.81	17.56	4.61	0.15	1.28	0.20	0.80	0.12	-

XRD analysis, shown in Figure 36, confirms the presence of CaCO_3 , among other important constituents MOOH, denoting a metal (M) hydroxide and MCO_3 , denoting a metal carbonate.

In this case, M represents either Fe or Mn.

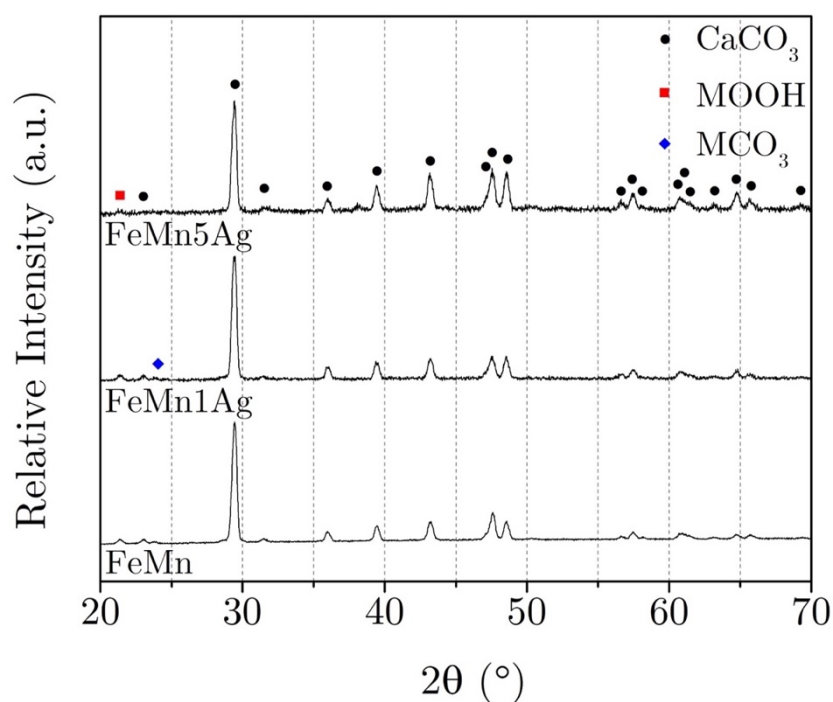


Figure 36 – XRD analysis of the explanted pins showing the presence of calcium carbonate and hydroxides and carbonates of Fe and Mn in all Fe-alloy pins.

4.6.2. Characterisation of the Pins after Sectioning

Analysis of the cross-sections of the explanted pins revealed the formation of a layer of corrosion product on their surfaces, as seen in Figure 37. This layer was variable in thickness but was noted to cover the entire surface of the pins. It was also noted to be consistently thicker in the FeMn5Ag implant specimens, where it reached a maximum depth of 30µm in areas.

EDS analyses, as shown in Table 35, revealed the presence of high amounts of C and O on the surfaces of the explanted pins. Along with the finding of Ca and P, this indicates the presence of organic material and bone on the pins' surfaces, this being intermixed with corrosion product. This finding is likely to signify the attachment of bone to the pins' surfaces.

In Figure 37(c), it can be seen that FeMn5Ag had a significantly thicker corrosion layer on its surface than the other materials did, being approximately 25µm at its thickest point in this case. The difference in EDS analysis results between points 3 and 4 confirms a higher amount of corrosion product in the form of oxide or hydroxide on the pin's surface. In addition, greater amounts of Ca and P, along with Na, K and C, in the superficial layer confirm the presence of organic material, this being intermixed with the corrosion products formed during the corrosion process of the pin.

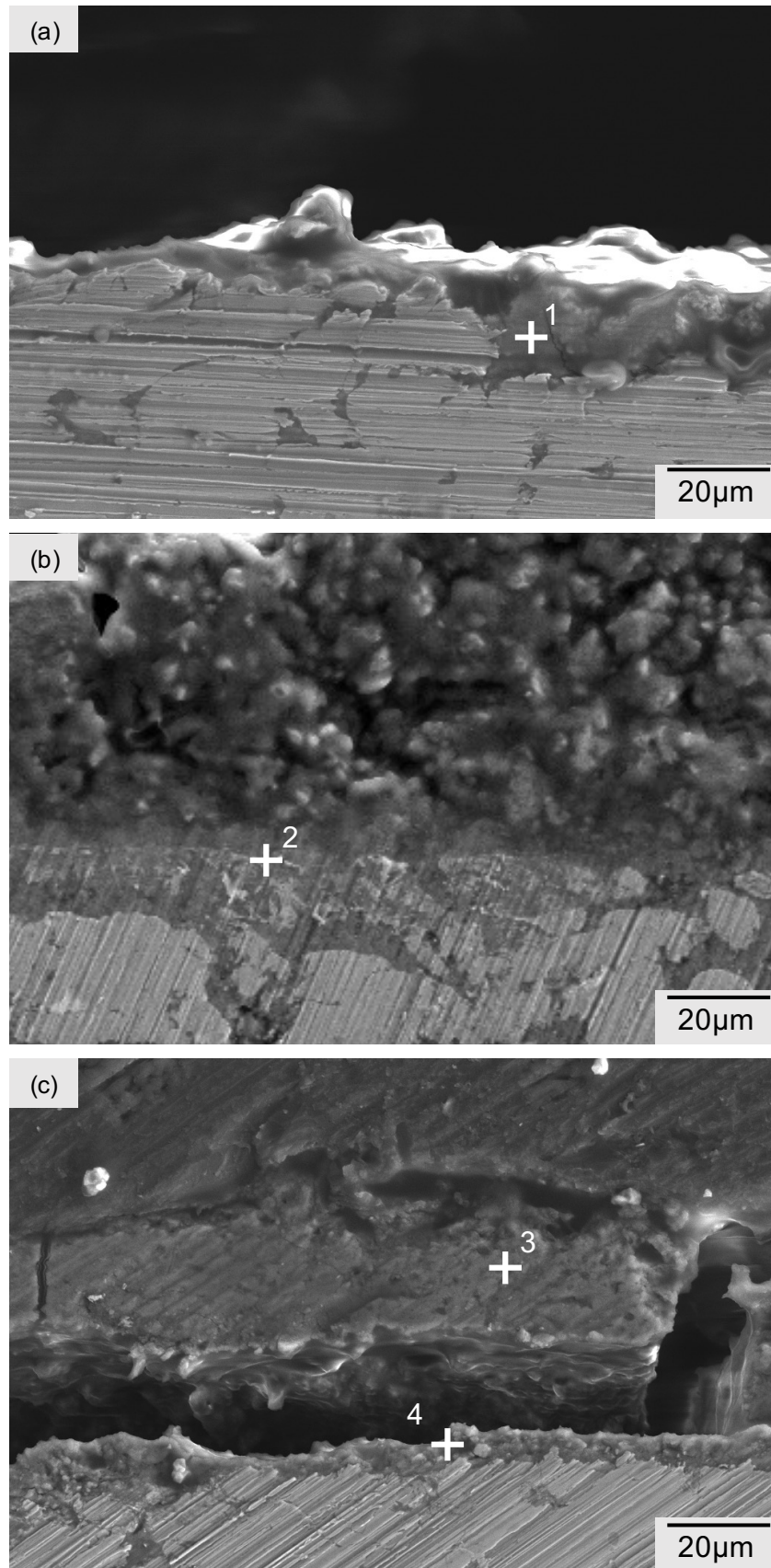


Figure 37 – High magnification SEM images of FeMn (a), FeMn1Ag (b) and FeMn5Ag (c) pins. Corrosion products are seen on their surfaces, this layer being thicker in the case of FeMn5Ag. EDS analyses of the cross-sections of the FeMn (1), FeMn1Ag (2) and FeMn5Ag (3, 4) pins were performed.

Table 35 – EDS analyses of cross-sections of the explanted FeMn (1), FeMn1Ag (2) and FeMn5Ag (3, 4) pins. The weight percentages of the detected elements are shown.

Element	Fe	Mn	O	C	Ca	P	K	Na	Ag
%Wt – 1	24.25	5.76	30.29	30.18	1.23	7.17	0.59	0.53	-
%Wt – 2	25.47	5.14	28.89	29.19	2.86	7.40	1.06	-	-
%Wt – 3	19.48	1.34	29.53	41.02	2.09	4.63	1.05	0.86	-
%Wt – 4	30.92	1.49	28.78	35.82	0.33	1.98	0.31	0.38	-

Discussion

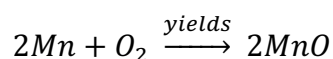
5.1. Characterisation of Materials Before Experimental Use

5.1.1. Composition and Microstructure of Sintered Coupons

The initial sintering process used for the manufacture of coupons and pins left a greenish, rough oxide layer on the surfaces of the Mn-containing coupons, as was the case with pure Fe coupons, albeit to a lesser extent. A grinding process was employed to remove this layer as the oxide would have interfered with their corrosion rate, altering their resultant cytotoxicity and biocompatibility. This process also exposed the porous architecture of the coupons and pins but was unable to remove the oxide products which had deposited within their pores (Figures 14, 15 and 16).

The H₂ present in the partially reducing atmosphere within the tube furnace is able to reduce Fe oxides, but this does not occur as easily with Mn. Mn has a very high affinity for O₂ and is known to sublime at the temperatures reached during the sintering process. As this happens, Mn reacts with the O₂ present in the partially reducing atmosphere to yield MnO, as in Equation 3.

Equation 3 – Reaction between Mn and O₂, yielding MnO.



The more O₂ that is present in the atmosphere during the sintering process, the more extensive the oxidation of Mn that occurs (Hryha, Dudrova et al. 2010, Hryha, Gierl et al.

2010). This explains the findings of Mn and O within the pores of the Mn-containing coupons before acid etching, as in Tables 18 and 19.

The portion of Mn that reacts with O₂ is therefore lost to the crystalline structure of the FeMn alloy. The austenisation of the material will subsequently depend on the portion of Mn which has not been oxidised. This Mn forms a solid solution with Fe and allows the formation of the face-centred cubic crystalline structure of γ -FeMn, which is stable at room temperature. Prevention of Mn oxidation is therefore important for the formation of γ -FeMn and so the use of a reducing atmosphere during the sintering process was considered critical (Caligari Conti, Mallia et al. 2019, Hryha, Dudrova et al. 2010, Šalak and Selecká 2012).

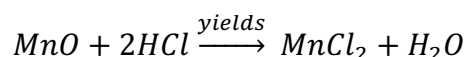
Mn also appeared to have been the reason for the difference in pore structure between materials. Most notably, pure Fe was seen to have less pores on its surface than the Mn-containing coupons. This was likely to be due at least in part, to the sublimation of Mn during the sintering process (Xu, Hodgson et al. 2017, Zhuang, Xu et al. 2020). Mn's lesser ductility than Fe was likely to be another contributing factor, allowing pores to remain open during the compression of the powders in the hydraulic press.

Other elements such as Al and Si were also found within the pores of coupons (Tables 17, 18 and 19). These elements may represent impurities in the powders used. In addition, Si may have come from the Silicon Carbide grinding paper used during the grinding process employed for all coupons. In addition, the OP-S suspension used for the final polishing step for those coupons subjected to SEM imaging and EDS analysis, is composed of a silica abrasive, contributing to the finding of Si.

5.1.2. The Effect of Acid Etching on the Composition of the Coupons and Pins

As mechanical means such as grinding are unable to reliably remove Mn oxides from within the pores of the structure, chemical means were required. In order to do this, a 1M solution of HCl was used, this concentration allowing the reaction to be controlled sufficiently to prevent damage to the coupons themselves. HCl reacts with the MnO present within the pores of the coupons, yielding MnCl₂ and water (H₂O), effectively removing the Mn oxide as per Equation 4.

Equation 4 – Reaction between MnO and HCl, removing MnO from within the pores of the coupons and pins.



This was the intended outcome, with SEM imaging of the Mn-containing coupons after acid etching showing empty pores, devoid of Mn oxide material (Figures 18, 19 and 20). EDS analysis also confirmed the removal of oxide material, with results obtained from pores in all cases of Mn-containing coupons being very similar to those obtained from the ground surface of the coupons (Tables 21 and 22). In contrast, SEM imaging and EDS results of pure Fe remained unchanged from those obtained before acid etching. In this case, amounts of O were similar both before and after the acid etching procedure (Figure 17 and Tables 20 and).

Removal of the oxide from within the porous structure of the coupons was considered important as its presence was very likely to have affected the outcome of subsequent *in vitro* and *in vivo* experiments. Firstly, Mn oxide may act as a barrier between the material underneath and the external environment, both in *in vitro* and *in vivo* settings. This should

slow corrosion rate and has been reported by several *in vivo* experiments (Čapek, Kubásek et al. 2016, Dargusch, Dehghan-Manshadi et al. 2019, Peuster, Hesse et al. 2006, Peuster, Wohlsein et al. 2001, Waksman, Pakala et al. 2008, Wu, Hu et al. 2012).

This however, is only likely to be a significant factor while the coupon or implant is totally covered by the oxide. In the absence of this, such as in a situation where Mn oxide is only present within the pores of a coupon, corrosion may actually be sped up. In this situation, MnO may act as a cathodic site (Yi and Majid 2018), thereby increasing the rate of corrosion of the remainder of the coupon. If this occurs, the oxides of Mn which are formed as a result of corrosion of the coupons and pins will once again form a barrier between the external environment and the uncorroded material beneath. Considering the thickness and microporosity of the coupons and pins tested, it is very likely that MnO will play a very minor role in the overall corrosion process. If it were to speed up corrosion, it would most likely only do so for a short period of time in the early stages of the process. For these reasons, it was decided to remove MnO from within the pore regions of the coupons and pins, thereby removing a variable of uncertain effect.

As the materials are exposed to water and air during the acid etching process, a corrosion inhibitor was required to prevent the re-accumulation of MnO. Hexamethylenetetramine (HMTA) was used to perform this function as it is known to adsorb onto the surface of metals exposed to acids, preventing their corrosion (Bayol, Kayakırlmaz et al. 2007). As a result of the addition of HMTA to the acid etching procedure, Mn-containing coupons and pins were free of MnO, both on their surfaces and within their pores (Figures 18, 19 and 20 and Tables 21 and 22). The coupons and pins' immediate use in *in vitro* and *in vivo* tests ensured that no time was available for the re-accumulation of MnO.

5.1.3. Crystalline Structure

Pure Fe at room temperature is present in its ferrite (α -Fe) form and has a BCC crystalline structure. It is able to form the FCC crystalline structure of γ -Fe (austenite) at higher temperatures, including those employed during the sintering process in this thesis. This phase is however unstable at lower temperatures and for this reason, it is only possible to obtain the ferrite form of pure Fe at room temperature, as has been the case here (Figure 21).

It is possible to stabilise the austenitic phase of Fe at room temperature by alloying with specific metals. Unlike covalent and ionic bonds, metallic bonds allow solid solutions of metals to form. This is only possible through the addition of metals which have similar crystalline structure and atomic volumes, Fe and Mn being two examples. In such cases, substitutional alloying occurs where Mn takes up positions within the crystalline structure of Fe. This changes the crystalline structure, making γ -FeMn stable at room temperature if adequate amounts of Mn are added (Pepperhoff and Acet 2001, Singh 2016).

If the metals are dissimilar with regards to crystalline structures and atomic volumes, as is the case for example with Fe (and Mn) and Ag, interstitial alloying occurs instead. In effect, this means that a solid solution is unable to form and Ag is therefore insoluble in Fe, Mn or FeMn. Ag is not able to occupy positions within the crystal lattice of Fe or FeMn and simply occupies spaces between them. This explains the formation of discrete clumps of Ag interspersed throughout the Ag-containing coupons (Figures 16 and 20 and Tables 19 and 22). In all cases, EDS analysis proved that Ag was not found within the general structure of the coupon but was confined only to these clumps (Caligari Conti, Mallia et al. 2019, Pepperhoff and Acet 2001, Singh 2016).

As Ag is effectively insoluble in FeMn and does not interfere with its crystalline structure, it allows γ -FeMn to maintain its FCC crystalline structure at room temperature, as has been

seen in this thesis (Figure 21). The desirable mechanical and antiferromagnetic properties of γ -FeMn are therefore maintained (Caligari Conti, Mallia et al. 2019, Dehghan-Manshadi, StJohn et al. 2019, Hryha, Dudrova et al. 2010, Hryha, Gierl et al. 2010, Pepperhoff and Acet 2001, Šalák and Selecká 2012, Singh 2016). Ag does however shift position during the sintering process as it melts at the temperatures reached during the sintering process.

5.2. *In Vitro* Cytotoxicity

5.2.1. Direct Versus Indirect Methods of Cytotoxicity Testing

The ISO 10993-5:2009, *Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity* specifies that indirect cytotoxicity testing techniques may be utilised instead of direct contact techniques. Despite this, it was decided to perform direct contact cytotoxicity tests for a number of reasons, but mostly because indirect methods may bias the results to show a favourable cytotoxicity profile.

The first reason for this is the reduced similarity of indirect methods to a real *in vivo* situation. Once a bone graft substitute is implanted *in vivo*, osteoblasts and other related cells would be expected to grow in direct contact with, and even through an osteoconductive implant. By definition, indirect methods of cytotoxicity testing do not reproduce this situation.

A second reason is that indirect experiments tend to make use of a relatively constant environment. Cells are exposed to elutions obtained by allowing the relevant materials to corrode within medium for specified periods of time. The material is then removed and cells from an established cell line are exposed to concentrations of the resulting elution. These elutions are therefore of a constant nature as they no longer contain an actively corroding material. This is very different to what is expected to occur in an *in vivo* environment where

the active corrosion of the bone graft substitute would be expected to produce a constantly changing environment.

In contrast to indirect cytotoxicity testing techniques, direct contact techniques allow the *in vitro* creation of an environment with more similarity to an *in vivo* situation. They directly expose cells to the dynamicity of an actively corroding material by expecting them to grow on the surfaces of the materials being tested. As a consequence of this, and because both *in vitro* and *in vivo* tests were performed in this thesis, it was thought necessary to perform *in vitro* cytotoxicity tests utilising direct contact methods.

Scarcello, Herpain et al. (2020) explained a possible reason for the differences in cytotoxicity seen between direct and indirect testing techniques (Scarcello, Herpain et al. 2020). In their experiment, this group showed the tendency of Fe-based materials to release hydroxyl radicals during their corrosion. This caused secondary oxidative stress in a dose-dependent manner, only to cells in direct contact with them. The same was not true for cells exposed in an indirect manner, these results tending to show a favourable cytotoxicity profile when compared to direct contact cytotoxicity tests. The use of catalase in the direct contact portion of their experiments relieved cytotoxicity, reinforcing this theory (Scarcello, Herpain et al. 2020).

Despite the benefits of employing direct contact over indirect contact techniques, all *in vitro* cytotoxicity tests are inevitably limited to some degree with regards to their ability to replicate a true *in vivo* environment. An *in vivo* blood supply, with different solute concentrations as well as actively replicating and replenishing cells, creates a dynamic nature which is not perfectly replicable in an *in vitro* situation.

This thesis aimed to target this issue on multiple fronts, the first of which has already been explained as the adoption of direct contact cytotoxicity testing techniques in favour of indirect

contact methods. The second, important facet of the adopted approach is the performance of a second set of cytotoxicity tests on pre-corroded coupons.

The biological replicate using an MC3T3-E1 cell line aimed to better mimic an *in vivo* situation through a fresh supply of bone-forming cells, after an initially rapid corrosion rate and its related cytotoxicity (Drynda, Hassel et al. 2015, Wiesener, Peters et al. 2017). As the coupons used in this test were pre-corroded for three days prior to their use in cytotoxicity tests, it was hoped that the corrosion rate might decrease to a level where cytotoxicity might be reduced, as seen in Figure 38. This however did not come to fruition with absolutely no cell viability being noted on any of the pre-corroded coupons, on any of the testing days. A similar result was also obtained for the as-ground, uncorroded coupons also used in this second set of tests ($P=.999$). Although it is impossible to exactly mimic the *in vivo* situation in an *in vitro* setting, this highlights the potential cytotoxicity of these materials used in direct contact testing methods. This additionally emphasizes the stress that Fe alloy materials may exert on cells in contact with them, as would be expected to occur in an *in vivo* environment (Scarcello, Herpain et al. 2020). Once again, the shortcomings of the indirect cytotoxicity testing method are brought into clearer focus.

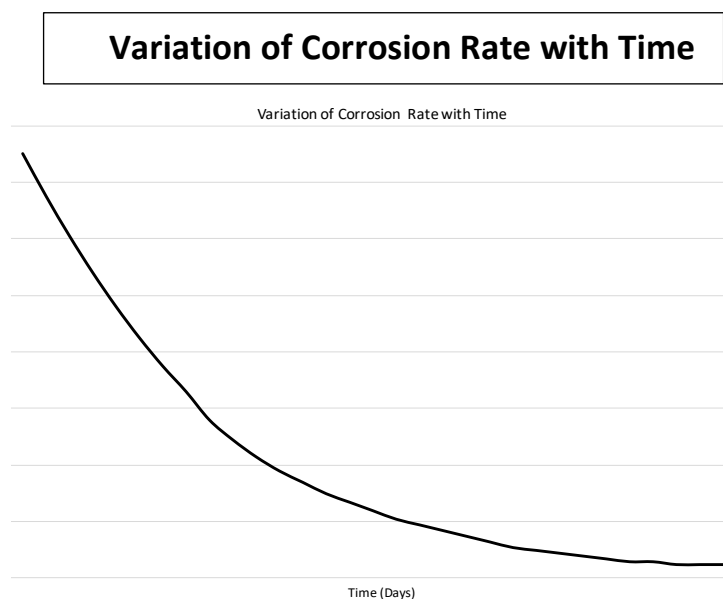


Figure 38 – Schematic plot depicting the expected decrease in corrosion rate of Fe alloy test materials over time.

5.2.2. Stainless Steel 316LVM and Thermanox™ Controls

In this experiment, performed via direct contact cytotoxicity testing methods, SS316LVM and Thermanox™ were used as positive controls. Both of these materials were seen to allow significant growth of both hFOB and MC3T3-E1 cells on their surfaces, evidenced by rising luminescence signals with increasing exposure times (Figures 22 and 23). SEM imaging and reflective light microscopy of SS316LVM also showed an increasing density of cells on its surface over the length of the test, further evidencing its appropriateness as a control material (Figures 24 and 25).

These results were expected since SS316LVM is routinely used in non-corroding metal implants within orthopaedic practice (Sansone, Pagani et al. 2013) and has evidenced no corrosion in this thesis (Table 32). In addition, recent literature has also seen this material used as a control (Moravej, Purnama et al. 2010). Thermanox™ on the other hand is specifically treated to allow the culture and adherence of cells on its surface.

Analysis of the results obtained in both hFOB and MC3T3-E1 tests revealed both similarities and differences between SS316LVM and Thermanox™ with regards to cell viability. On one hand, both showed increasing cell viability over the four-day test period with a relatively smaller increase in viability between days one and two than between days two and four. This was more obvious in the MC3T3-E1 tests and occurred firstly because of the greater time gap between days two and four than between days one and two. In addition, once cells adhered to the coupons' surfaces and began to replicate, they did so exponentially, reaching a log phase of growth as confluence increased. The seeding concentration of 30,000 cells/ml for hFOB cells and 15,000 cells/ml for MC3T3-E1 cells was in fact specifically chosen after pre-cytotoxicity testing trial experiments had been run. These concentrations prevented cells from becoming over-confluent and introducing a source of error.

An important difference between the viability results obtained for these materials was the apparent greater viability of SS316LVM in both hFOB and MC3T3-E1 tests. In actual fact, this was not likely to have resulted from a difference in biocompatibility of these materials, but rather a simple difference in surface area available for seeding of cells. As the Thermanox™ coverslips are very thin, they were raised off the bottom of the well plates. This ensured that the same volume of medium (containing cells) was present over each Thermanox™ coverslip as it was over the SS316LVM coupons. Despite this attempt to avoid error, this did not address the approximate 4mm difference in height that existed between the Thermanox™ coverslips and SS316LVM coupons. This difference created an advantage for SS316LVM as it simply had more available surface area for cell attachment, even when considering the small excess edge left on the Thermanox™ coverslips which allowed for their handling.

5.2.3. *In Vitro* Cytotoxicity of Test Iron Alloy Materials

In order to better understand the luminescence results obtained from the cell viability tests performed using the CellTiter-Glo® viability assay, an unseeded medium negative control was used in addition to the seeded test Fe alloy coupons. The luminescence results obtained from the negative control well gave a reading of background luminescence and acted as a reference against which both positive controls and test Fe alloys could be compared.

The obtained results clearly indicated that all Fe alloy test materials were significantly cytotoxic (Figures 22 and 23). After four days of exposure to these materials, the obtained luminescence signals were similar to those obtained for the medium only negative controls. This was the case in both hFOB and MC3T3-E1 tests, indicating the absence of any viable cells growing in direct contact with the test Fe alloy coupons. SEM imaging and reflective light microscopy further confirmed this result in the case of the MC3T3-E1 tests, showing a complete lack of any cells growing on their surfaces (Figures 24 and 25).

The only test material to have shown any potentially viable cells growing on its surface was pure Fe. After one day of exposure to pure Fe during the hFOB cytotoxicity tests, this material showed luminescence signals above those obtained for the negative controls (Figure 22). This suggests that there may have been viable cells growing on the surfaces of these particular coupons at this time. Closer analysis however revealed a large standard deviation for this result, indicating that this may have been due to error (Table 24). In addition, viability was still much poorer for pure Fe than it was for the positive controls. Results obtained after a further day of exposure to this material (two days of exposure in total), confirmed that any cells growing on the pure Fe coupons had expired. In this case, luminescence results were similar to those obtained for the medium only negative control.

Initial viability may have been due to the recognised, lesser cytotoxicity of pure Fe than of Mn-containing Fe alloys (Hermawan, Purnama et al. 2010), but may also be explained by a second theory. Acid etching was seen to remove any Mn oxides within the porous structures of the Mn-containing coupons. This exposed more material to corrosion and may therefore have increased cytotoxicity. Pure Fe was contrarily unaffected by this procedure, ergo these coupons retained more oxides within their porous structures. The result of this was the presentation of a lower total surface area available for corrosion.

An important observation was made for the Ag-containing coupons in the hFOB cytotoxicity tests. These coupons appeared to exhibit a significantly decreasing luminescence with an increasing exposure time (Figure 22 and Table 24). One potential explanation for this is the decreasing viability of cells exposed to these materials. This is unlikely to be the true explanation for this observation however, as luminescence is comparable to that of the medium only negative controls, suggesting the absence of any viable cells. The more likely explanation is the flaking of insoluble corrosion products from the surfaces of these particular coupons.

As the flaking of insoluble corrosion products was expected, the coupons were only rinsed after the transfer of the coupons to fresh 6-well plates. This ensured that any cells present on the flaking material underwent the trypsinisation procedure, and therefore contributed to the result. The second reason for the transfer of coupons was to prevent erroneous readings from cells growing on the well bottom and side-walls. The problem with flaking material is that although it may not be macroscopically visible, it may still find its way into the CellTiter-Glo® assay wells, decreasing the transmittance of luminescence signals from the well. Although this was unlikely to have affected the overall results with regards to cell viability, it may have lowered the luminescence signals obtained. This effect is more likely to be seen with longer

exposure times, as occurred in this case. Inferentially, this may also be the result of a greater amount of corrosion occurring with the Ag-containing coupons.

These results provide a stark contrast to most of the currently available *in vitro* evidence, but the fact that this is for the most part performed using indirect contact cytotoxicity techniques (Caligari Conti, Aquilina et al. 2018, Čapek, Kubásek et al. 2016, Dargusch, Dehghan-Manshadi et al. 2019, Hermawan, Purnama et al. 2010, Moravej, Purnama et al. 2010, Nie, Zheng et al. 2010, Nie, Chen et al. 2021, Sharma, Jain et al. 2020), must be acknowledged. This evidence tends to portray pure Fe and FeMn as having minimal cytotoxic effect as judged by the *ISO 10993-5:2009, Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity*.

Relatively less literature is available regarding experiments performed using direct contact cytotoxicity testing techniques (Nie, Zheng et al. 2010, Nie, Chen et al. 2021, Sharma, Jain et al. 2020, Shuai, Yang et al. 2020). One such study was performed by Shuai, Yang et al. (2020). In this work, both direct and indirect contact cytotoxicity testing techniques were performed using pure Fe and Fe₂₅Mn scaffolds. These had porosities of approximately 66.7% and were produced through a process of selective laser melting. This group evidenced an increased corrosion rate of the Fe₂₅Mn scaffolds as compared to those of pure Fe. In this experiment however, FeMn existed in all of α , γ and martensitic phases, the potential differences of which were likely to have contributed to the increased corrosion rate seen with these implants (Shuai, Yang et al. 2020). The increased corrosion rate, coupled with the increased porosity of approximately 66.7%, should have increased cytotoxicity, but this does not appear to have been the case and contrasts with the results obtained in this thesis.

A more similar experiment was performed more recently by Nie, Chen et al. (2021). This work dealt specifically with γ -Fe₃₀Mn in direct contact cytotoxicity conditions, very similar to

the work performed in this thesis. Despite the similarities, the end result was different to the results obtained here. Nie, Chen et al. (2021), similar to Shuai, Yang et al. (2020), also show FeMn in a positive light, having caused minimal cytotoxicity to the MC3T3-E1 cells seeded onto the scaffolds (Nie, Chen et al. 2021, Shuai, Yang et al. 2020).

One possible explanation for the differences seen between these two studies and this thesis is provided by the size of the samples tested. In the latter experiment by Nie, Chen et al. (2021), 2 x 6mm pins were used while the scaffolds used by Shuai, Yang et al. (2020) were of sufficiently small size to fit into 24-well plates (Nie, Chen et al. 2021, Shuai, Yang et al. 2020). In contrast, the coupons used in this thesis' work were cylinders of 21.8mm diameter and 4mm depth, requiring significantly larger 6-well plates for testing. This undoubtedly increases the surface area and volume of material available of corrosion, especially considering that the coupons are porous. The larger amount of exposed, uncorroded material present in this thesis' work should therefore allow for the rapid formation of a large amount of corrosion products, at least in the initial stages of corrosion. This is likely to have affected the viability results obtained in this work, which contrast significantly with much of the available literature (Nie, Chen et al. 2021, Shuai, Yang et al. 2020).

Considering this, it must be emphasised that one of the intended uses for corroding bone graft substitutes is in situations where autografted bone is not available in sufficient quantities, necessitating the use of large amounts of bone graft substitute.

One must also consider that corrosion rate is likely be fastest at the start of the experiment with eventual slowing of corrosion over the following days. This is exemplified in the *in vivo* portion of experiments performed here where the pins explanted after six months of exposure to an *in vivo* environment showed corrosion mostly limited to their outer parts, with a maintained internal structure (Figure 37). This has also been seen and explained by other

groups for both *in vitro* and *in vivo* environments (Drynda, Hassel et al. 2015, Wiesener, Peters et al. 2017).

An important result was obtained by Sharma, Jain et al. (2020). In their experiment, different structures of pure Fe were shown to corrode at different rates with this difference translating into changes in cytotoxicity. Although indirect cytotoxicity tests showed significant cytotoxicity, with the more rapidly corroding material found to be the most cytotoxic, direct contact tests proved even more cytotoxic with almost no cells remaining on the surfaces of the scaffolds by the end of the testing period (Sharma, Jain et al. 2020). This observation adds further weight to the findings of Scarcello, Herpain et al. (2020) that cells growing in direct contact with Fe-based materials may suffer from oxidative stress as a result, versus those cells only exposed in an indirect manner to the test materials (Scarcello, Herpain et al. 2020).

Although indirect cytotoxicity techniques were used by Caligari Conti, Aquilina et al. (2018), their findings add further weight to the observation that cells exposed directly to either corroding materials or their insoluble corrosion products suffer increased cytotoxicity. In their experiment, a potentiostatic elution of Fe₁₃Mn_{1.2}C was created, this containing particulate material. Cells were then exposed either directly to this elution or a centrifuged portion of it, with results clearly indicating a better cell viability in the centrifuged elution (Caligari Conti, Aquilina et al. 2018).

The little evidence that is currently available for Ag-containing Fe alloys is once again performed using indirect cytotoxicity techniques. Nevertheless, the literature is conflicting with some reports suggesting that a higher Ag content in an Fe₃₀Mn alloy may cause lesser toxicity as compared to a lower Ag content, or simply FeMn (Bagha, Khakbiz et al. 2020, Liu, He et al. 2019). Still others suggest the contrary, with increasing cytotoxicity being exhibited by higher Ag content materials (Sotoudehbagha, Sheibani et al. 2018). These results are, once

again, not directly comparable to the results obtained here, primarily because of the difference in testing techniques. In addition, the results obtained in this thesis only show that pure Fe is potentially less cytotoxic than FeMn, FeMn1Ag and FeMn5Ag, at least for hFOB cells. It cannot be said which of the latter three is most cytotoxic however as results were similar across all testing days and between both cell lines with no viability having been recorded.

Perhaps the most similar work was performed by Robles Cuellar (2021) who performed both direct and indirect cytotoxicity tests on pure Fe, Fe35Mn, Fe35Mn1Ag and Fe35Mn5Ag. The coupons used in this particular work were however smaller in size and were not acid etched. Robles Cuellar showed a significant cytotoxic effect of all the test Fe alloy materials in indirect cytotoxicity tests. The cytotoxicity was however even more pronounced in direct testing, concordant with the results obtained in this thesis (Robles Cuellar 2021).

5.3. *In Vivo* Biocompatibility

5.3.1. Lost Pins

Although a total of twenty-four test Fe alloy pins had been implanted into the tail vertebrae of test rats at the start of the *in vivo* portion of experiments, only thirteen were found to still be present at the end of the six-month implantation period (Figure 30 and Table 33). There are only two possible explanations for the disappearance of eleven of the implanted pins, the first being the complete corrosion of the implants within this six-month period. The second explanation involves the removal of the implant from its position, either through removal by the rat itself, or through loosening of the pin to come to lie in a different position.

Considering the results obtained from the implanted pins in the other rats who retained the implants for the totality of the six-month implantation period, the second explanation is the most plausible. The thirteen retained pins showed very little signs of corrosion overall, suggesting that corrosion rate was not rapid enough for the entire corrosion of the eleven lost pins (Figures 34 and 37 and Table 35). In addition, CT imaging which included whole sections of tails was performed, encompassing surrounding vertebrae in addition to the vertebrae where the implants were initially positioned (Figure 30). This imaging modality did not show the implants to be dislodged and lying within the surrounding soft tissues, which themselves should have limited the migration of the pins, adding further weight to the hypothesis that the implants had been lost entirely.

It is only possible that the implants had been lost soon after their implantation. Allowing any significant amount of time to elapse after implantation will have meant the incorporation of the implants into surrounding bone, as evidenced by the difficulty in removing them after the six-month implantation period. The healing of the surrounding soft tissues including tendon and skin, would also have prevented the removal of the implant. In addition, blackening of the surrounding bone would have been expected, as was seen in histological specimens where pins were retained (Figures 31 and 32). This was not seen on histological examination of specimens taken from tails with lost pins (Figure 33).

Each rat with a lost implant must also have removed its own implant as each of the twenty-four test animals were kept in their own individual cages with no possibility of contact between rats. While unfortunate, this phenomenon was seen as a possibility during the design stages of this experiment. This is in fact the first reason for choosing a vertebra close to the base of the tail within which to implant the pins, as this makes it more difficult for the rat to reach the surgical site. The second reason is the size of the bone required to accommodate

the implant, vertebrae at the base of the tail being larger and more accommodative of an implant. The tendency of rats to auto-manipulate their surgical sites has also been recorded by other groups (Drynda, Hassel et al. 2015). While the rats were not directly seen to manipulate their own wounds, this must have happened during periods of time when they were not under observation. Perhaps this could have been avoided through the adoption of other preventative measures such as the use of a neck cone in the early post-operative period, though this would undoubtedly have brought further discomfort to the rat. The final proof of the rats tampering with their own surgical sites is that each had removed their own sutures by the time it was necessary to do so at ten days post-operatively.

5.3.2. Incorporation and Biocompatibility of the Explanted Pins

Despite the loss of eleven of the twenty-four implants, the thirteen remaining implants were of different compositions with FeMn, FeMn1Ag and FeMn5Ag all being represented and analysed (Table 33). As the biocompatibility of pure Fe is already well documented and its characteristics with regards to corrosion rate, resistance and magnetic properties are not desirable, it was thought reasonable to forego experimentation using implants made purely of this element (Peuster, Hesse et al. 2006, Peuster, Wohlsein et al. 2001, Waksman, Pakala et al. 2008, Wu, Hu et al. 2012).

The results obtained in this thesis with regards to *in vivo* biocompatibility are promising. None of the rats showed any signs of stress or ill health during the six-month implantation period, with all of the rats reaching the end of the experiment. Macroscopic examination proved all implants had become well fixed within bone and were not easily removed, suggesting that they were acceptable to their environment, at most causing a level of cytotoxicity which the surrounding tissues were able to overcome. This fits well with the

evidence available for both FeMn and FeMn1Ag implants positioned within the bones of animals (Dargusch, Venezuela et al. 2021, Nie, Chen et al. 2021).

The finding of incorporation of the implants within bone is similar to the reports by other groups such as Traverson, Heiden et al. (2018). In their experiment, histological analysis of bone surrounding a Fe30Mn implant revealed a large area of contact between the implant/corrosion surface and surrounding bone. Analysis however also revealed areas of bone necrosis and histological appearances of inflammation, as seen in Figure 39. This resulted in their classification of this material as a mild local irritant and contrasts somewhat with the results obtained in this thesis (Traverson, Heiden et al. 2018). Carluccio, Xu et al. (2020) also evidence good bone growth onto the implant/corrosion surface (Carluccio, Xu et al. 2020). This is a finding which is closely mirrored by this thesis, which evidences no signs of inflammatory change or necrosis.

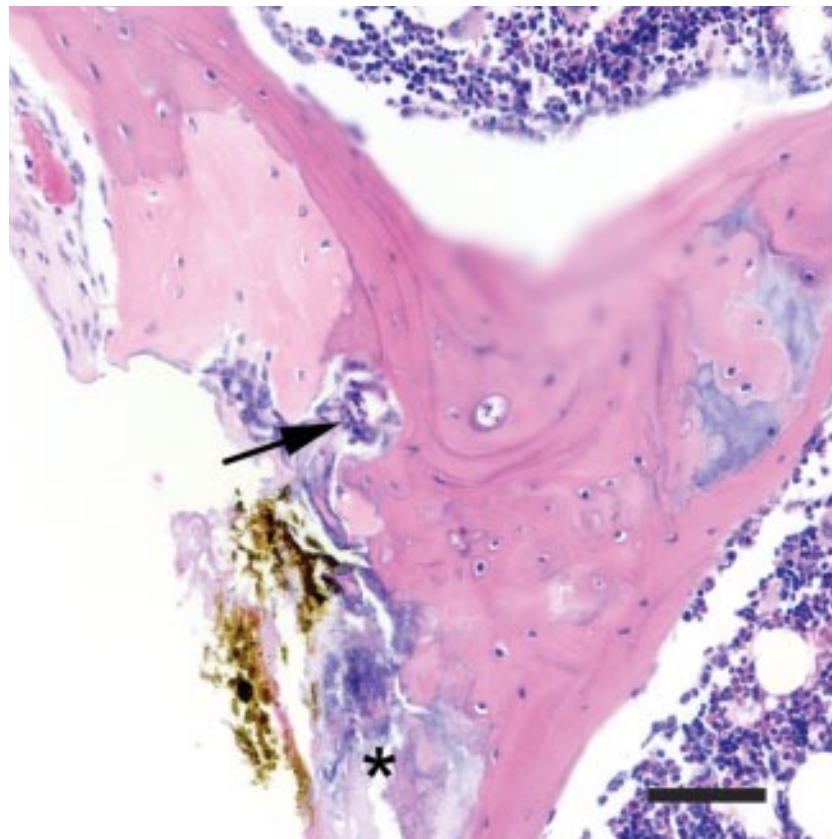


Figure 39 – Histological image of bone surrounding an implanted Fe30Mn wire, this being removed before histological examination was performed. Areas of bone necrosis (star) are noted in close proximity to corrosion products, along with inflammatory giant cells (arrow). The bar represents 100µm. This image is adapted from Traverson, Heiden et al. (2018) (Traverson, Heiden et al. 2018).

Microscopic examination of the explanted pins in this thesis showed that the porous structure towards the surface of the pins had filled with corrosion product, and did not contain any cellular or bony material (Figure 37). This was not unexpected as the pore sizes were in general, smaller than would be expected to allow for osteoconduction (Chang, Hong et al. 2000, Tsuruga, Takita et al. 1997). Despite this, EDS analysis of the implanted pins' surfaces and surrounding areas showed an increasing amount of Ca and P with increasingly superficial examination (Figures 35 and 37 and Tables 34 and 35), along with an XRD finding of what was likely to be CaCO_3 (Figure 36). While it must be kept in mind that EDS analysis is limited with

regards to the detection of light elements such as C and P, this finding likely signifies the adhesion of calcified bone matrix and cellular material to the outer surface and corrosion layer of the implant. This is strengthened further by the histological finding of mature bone formation which was similar in architecture to that of control bone (Figure 31). This resonates with what has been seen in other works, which have also evidenced good incorporation of similar implants within bone (Carluccio, Xu et al. 2020, Traverson, Heiden et al. 2018).

Another explanation for the finding of higher levels of P, which outweighed Ca on the outer surfaces of the implants, is the formation of passivation layers in the form of Fe and Mn phosphates. These compounds, along with oxides of Mn and to a lesser extent of Fe, are the likely explanation for the macroscopic and histological blackening of bone surrounding the pins (Arroyo, Ruiz et al. 2008, Shahack-Gross, Bar-Yosef et al. 1997, Stathopoulou, Theodoropoulou et al. 2013, Waksman, Pakala et al. 2008). In addition, Fe and Mn phosphates along with the formation of insoluble corrosion products on the surfaces on the pins, are the most likely explanation for the low overall corrosion seen in the pins after the six-month period of implantation. These findings have also been noted by other groups (Drynda, Hassel et al. 2015, Wegener, Sichler et al. 2020), and along with the decreased availability of O in the area, are likely the reason for some groups' findings of decreasing *in vitro* and *in vivo* corrosion over time (Kraus, Moszner et al. 2014, Tonna, Wang et al. 2022).

In turn, this finding may in part explain the reason for the favourable biocompatibility results obtained, as opposed to the *in vitro* findings of significant cytotoxicity. As the formation of passivation layers in an *in vivo* environment shields deeper, uncorroded material from exposure, slowing the resultant corrosion rate, cytotoxicity is likely to improve over the same time period. Although this may occur to an extent in an *in vitro* situation (Tonna, Wang et al. 2022), it is likely to be more profound *in vivo*, where newly-forming bone and the surrounding

soft tissues prevent the movement of flaking corrosion products. This in turn prevents exposure of uncorroded material underneath the corrosion products, preventing a re-acceleration of corrosion rate and allowing the maintenance of a low cytotoxicity. Further proof of this is the negative findings of Fe products within vital organs such as the hearts and livers of rats within this thesis. This finding in particular evidences good biocompatibility *in vivo* at the expense of corrosion rate.

While some flaking was in fact seen in cross-sectional SEM images of the pins in this thesis (Figure 37), this was most likely to have occurred during manipulation of the implant during and after extraction. The majority of the visualised corrosion products had in fact remained closely adherent to the surfaces of the pins. In contrast, the *in vitro* environment afforded by a direct contact cytotoxicity testing method may have allowed for flaking of insoluble corrosion material from the surfaces of the tested alloys, this impacting cytotoxicity in its own right (Caligari Conti, Aquilina et al. 2018). In addition, this likely exposed fresh, uncorroded material while allowing a renewed supply of O to the site. It is probable that this re-accelerated corrosion and resulted in a once again increased cytotoxicity.

A second plausible explanation for the difference between *in vitro* and *in vivo* results with regards to cytotoxicity and biocompatibility lies with the dynamic cellular nature of an *in vivo* environment, as compared to an *in vitro* environment. The *in vitro* situation employed here made use of differentiated cell lines with absolutely no possibility of a fresh supply of osteoblasts or their precursor cells. Consequently, any depleted cells were not replaced, meaning that the advantage of a decreasing cytotoxicity over time could not fully be taken advantage of. This was not so in the *in vivo* environment where a supply of mesenchymal stem cells (MSC), provided through local and systemic means, as well as osteoblasts through surrounding periosteum and bone, ensured that cells were replaced as they were lost.

Consequently, as passivation layers formed on the surface of the implant with a resultant slowing in the implant's corrosion rate and lowering in cytotoxicity, the balance may eventually have shifted in favour of formation of bone.

This fact may render the appearance of the *in vivo* environment advantageous, but this may not be the case in all situations of trauma for example. Trauma situations are varied and concern patients of all shapes and sizes but more importantly, different health statuses. The ability to incorporate a graft with inherent cytotoxic qualities in a patient who is in poor health may become very limited. This is especially true when taking patients of previous poor nutrition, with associated co-morbidities such as diabetes mellitus into account, in the setting of major trauma with associated bone loss. Adding to this the possibility of infection in open fractures, one may begin to understand the limitations of corroding Fe alloy grafts in such a scenario, emphasizing the decision making process of the surgical team with regards to the use of these implants in cases of this nature.

Another consideration is that the *in vivo* environment utilised a changing extracellular fluid, with the ability to wash away and excrete any toxic corrosion products which dissolved in it. This did not occur in the *in vitro* experiments performed in this thesis and undoubtedly affected the results.

For the most part, the current implants exhibited microporosity, with only a small number of pores exceeding 50µm in size. This was likely to be a major contributor to the low overall corrosion rate, while sparing cytotoxicity. In order to manipulate this balance in favour of increasing corrosion rate, more weight needs to be placed on increasing the surface area available for corrosion. Increasing the porosity through larger pore sizes and improved pore interconnectivity is very likely to aid in this regard, as it should increase the osteoconductive nature of the graft while also increasing the exposed surface area. In addition, it should allow

for the evacuation of accumulated corrosion products, preventing their passivating effect and decreasing the depth of material. This in turn, will increase surface area to volume ratio.

Although manipulating the implants in such a manner is likely to benefit corrosion rate, it may on the contrary negatively affect their capacity to adequately perform in load bearing orthopaedic applications. One study by Wegener, Behnke et al. (2021), employed the creation of a defect within the tibiae of sheep, where an Fe-based implant was sited. This group evidenced a high rate of corrosion with corrosion particles being found within regional lymph nodes. It must be noted however that the implants used had porosities in the region of 85%, rendering them unlikely to withstand prolonged weight-bearing orthopaedic applications (Wegener, Behnke et al. 2021).

The necessity of these implants to function in load-bearing applications was thought of as essential and was one of the reasons behind the structure chosen. Increasing porosity and pore interconnectivity has the effect of decreasing the rigidity of the implant, which may therefore fail if significant load is applied. In the *in vivo* situation employed in this thesis, this was not possible as the implants were implanted within a controlled defect within bone and were therefore not subjected to significant load. Nonetheless, the eventual implant is destined to function in such environments and so it was thought reasonable to maintain a lower overall porosity. There is one design flaw however, this being the microporosity of the implant rendering it unable to effectively allow bone to grow through it (Figure 37 and Table 35).

Comparatively, the findings of this thesis fit well with the available literature, despite there being a relative paucity of evidence in this area. The little evidence that is available is made even scarcer by the decision of some groups to position the implants not within bone, but within subcutaneous soft tissues instead. Drynda, Hassel et al. (2015) performed an

experiment which dealt with the *in vivo* biocompatibility of FeMn alloys with Mn forming between 0.5% and 6.9% of the final weight of the alloy. In their experiment, the final alloys were positioned within the subcutaneous tissues of rats, close to the hip region (Drynda, Hassel et al. 2015). This limits the comparability of this experiment to that performed in this thesis in two major ways, the first being the composition of the implants.

The experiment performed by Drynda, Hassel et al. (2015) deals with alloys containing amounts of Mn which are well below those used in this thesis. This may mean that the alloys utilised contained larger amounts of α -FeMn and relatively less of the austenitic phase of the material. In addition, *in vitro* experiments clearly showed that Mn causes more cytotoxicity than Fe, increasing the likelihood that an implanted device with higher concentrations of Mn, as were used in this thesis, will cause issues with biocompatibility (Drynda, Hassel et al. 2015, Hermawan, Purnama et al. 2010, Liu and Zheng 2011).

The second limitation stems from the implantation of the alloys within subcutaneous tissues. It is true that an implant of this nature is very likely to come into contact with tissues other than bone when implanted within a human and therefore its acceptability to these tissues is important. Despite this, its primary function is to allow the attachment of bone to its surface and facilitate the formation of bone within its structure, meaning that this situation is not a true representation of the implant's eventual use. Despite these differences and limitations, this experiment showed FeMn alloys to have good biocompatibility *in vivo* (Drynda, Hassel et al. 2015).

Another experiment concerning the *in vivo* implantation of FeMn alloys was performed by Nie, Chen et al. (2021). In this experiment, Fe₃₀Mn implants with both α and γ phases were implanted into the femora of rabbits. In comparison to the mostly microporous structure of the implants used in this thesis however (Figures 14, 15 and 16), the implants used by Nie,

Chen et al. (2021) were characterised to have pore sizes of up to 600µm. This group achieved favourable biocompatibility results and were able to show the ingrowth of bone into the porous structure of the material, this not being possible in this thesis considering a pore size mostly around the 50µm mark (Chang, Hong et al. 2000, Nie, Chen et al. 2021, Tsuruga, Takita et al. 1997). Nevertheless, the results obtained in this thesis with regards to biocompatibility are similar, with histological examination evidencing the formation of mature bone (Figures 31 and 32). Much of the remaining *in vivo* literature concerning FeMn alloys is similar in that it deals with Mn content in the region of 30-35% by weight and tends to show similarly good biocompatibility, at the expense in most cases (as in this thesis), of corrosion (Carluccio, Xu et al. 2020, Traverson, Heiden et al. 2018, Wegener, Behnke et al. 2021, Wegener, Sichler et al. 2020).

Dargusch, Venezuela et al. (2021) performed a similar *in vivo* work, this apparently being the only other work studying FeMnAg alloys in an *in vivo* environment. In their work, implants were fashioned from Fe35Mn and Fe35Mn1Ag and implanted within soft tissues. They evidence an increased corrosion rate for the Ag-containing implants in comparison with Fe35Mn but also showed a greater amount and rate of gas formation in the former. Despite these findings, the overall outcome was the same, this group reporting acceptable biocompatibility for all implants (Dargusch, Venezuela et al. 2021).

5.4. The Effect of Silver

5.4.1. Biocompatibility

Dargusch, Venezuela et al. (2021) have performed the only other work evidencing biocompatibility for Ag-containing Fe-based alloys. In their work, Fe35Mn and Fe35Mn1Ag

implants were positioned within the soft tissues of rats. Although this group evidences an increased rate of corrosion for the Ag-containing implant, the amount and rate of gas formation is also greater in relation to this material. The result is however unchanged, with both FeMn and FeMn1Ag implants evidencing good biocompatibility (Dargusch, Venezuela et al. 2021).

This work is not directly comparable to the work performed in this thesis, in view of this group's decision to position the implants within soft tissues and not in bone. Despite this, this thesis evidences good biocompatibility in bone for the Ag-containing implants. Another similarity is seen in the apparent corrosion of the implants used in this thesis (Figure 37). Although not quantified directly, FeMn5Ag evidently had a thicker layer of corrosion than either FeMn or FeMn1Ag. This would suggest that Ag increases the corrosion rate of *in vivo* implants of this nature, without having a significant impact on biocompatibility. This also fits well with *in vitro* findings of increased overall corrosion of the FeMn1Ag and FeMn5Ag coupons' surfaces (Figure 28).

5.4.2. Corrosion Rate

There is as yet little evidence regarding the corrosion rate of Ag-containing Fe alloys in the literature. Consequently, it is not completely clear whether Ag accelerates corrosion rate or not (Bagha, Khakbiz et al. 2020, Sotoudeh Bagha, Khakbiz et al. 2018, Sotoudehbagha, Sheibani et al. 2018, Tonna, Wang et al. 2022, Wang, Tonna et al. 2022). The *in vitro* results obtained in this thesis do not provide a clear answer to this question but may suggest that Ag does in fact have the potential to increase corrosion rate. This is evidenced by the increased corrosion present over the surfaces of the Ag-containing coupons on SEM imaging (Figures 26, 27 and 28), and is supported by the findings of increased amounts of O on EDS examination

of the general surfaces of these coupons as compared to pure Fe and FeMn (Tables 29, 30 and 31). Despite this, the exact amount of corrosion is not quantified in this thesis and therefore it is impossible to say with certainty that Ag increases the corrosion rate of Fe alloys in an *in vitro* scenario.

In addition, as the implant corrodes and a layer of corrosion product forms on its outer surface, both uncorroded FeMn material as well as Ag clumps are likely to lose contact with the outside environment. This was evidenced in this thesis by the absence of Ag after testing on both SEM imaging and EDS analysis of *in vitro* coupons (Figures 26, 27 and 28 and Tables 29, 30 and 31) and *in vivo* implants (Figures 35 and 37 and Tables 34 and 35). The micro-galvanic effect of Ag is likely to be lost as this happens, once again limiting corrosion.

The situation is similar for *in vivo* Ag-containing Fe alloy implants. SEM and EDS analysis of the explanted pins only showed a small amount of corrosion of the surfaces of the pins after six months of *in vivo* implantation. FeMn5Ag seemingly exhibited thicker layers of corrosion product, reaching a maximum of 30µm depth in certain areas. This may in fact be evidence of an increased corrosion rate for Ag-containing implants and was likely to be a result of the micro-galvanic currents created (Bagha, Khakbiz et al. 2020, Liu, He et al. 2019, Tonna, Wang et al. 2022). In spite of this, it was expected that the amount of corrosion would be greater than was actually seen and so it cannot be stated with absolute certainty that the addition of Ag does increase corrosion rate *in vivo*.

Once again, the formation of a passivation layer was most likely to be the major contributing factor to the slow corrosion rate, as evidenced by the P content on the surfaces of the pins (Figure 37 and Table 35). A closer look at these images also shows the relatively untouched internal structure of the pins. It is evident that corrosion was limited to their outer surfaces, this likely to have occurred as a result of the reasons mentioned. The small pore size

with ease of clogging with corrosion products, along with the high volume to surface area are very likely contributors, as was the tendency of the accumulated, insoluble corrosion products to remain in close proximity to the underlying implant's surface, contrasting with the findings of Wegener, Behnke et al. (2021). It must be re-iterated however that the work by Wegener, Behnke et al. (2021) deals with structures that are not likely to hold up well in load-bearing orthopaedic applications. Even so, their implants failed to corrode sufficiently over the *in vivo* study period of twelve months (Wegener, Behnke et al. 2021).

5.5. Potential of Iron-Manganese and Iron-Manganese-Silver as Implantable Bone Scaffolds

Although the FeMn and FeMnAg alloys used in this thesis are biocompatible, they are not currently fit for use as corroding bone scaffolds. The first reason for this is that neither of the implanted pins showed any significant amount of corrosion over the six-month implantation period. It was hoped that the addition of Ag might increase corrosion rate to a more significant degree but this was unfortunately not the case. The result of this in a real patient scenario would likely be the incorporation of the graft into surrounding bone, without any significant bone growth through the implant. For this reason, the implants may actually act as areas of weakness as no true bond exists between them and surrounding bone. Fractures may be more likely to occur in these regions as a result.

In addition, despite Ag's potential antibacterial properties, the long-term presence of a non-corroding implant may serve as a potential nidus for infection. If this occurs, the surrounding of such an implant with bone may have disastrous consequences for the patient.

The need to remove such an implant would mean the destruction of large quantities of bone, the very problem that this implant set out to solve.

While there exists promise in these material's capabilities, there are lacunae in several aspects of their development and function. Their manufacture needs to be further developed to be able to accurately control their structure, including their porosity and pore interconnectivity. In turn, this will influence their corrosion rate, cytotoxicity and structural integrity. The correct balance between these three critical elements has not yet been achieved with Fe alloy materials.

Conclusions

1. The powder metallurgy process used to manufacture the Fe alloy coupons had a great impact on their resultant composition and structure. The attainment of the austenitic phase of FeMn in FeMn, FeMn1Ag and FeMn5Ag materials was made possible through the addition of Mn and the sintering process employed. As a result of this, changes were also observed in the porous structures of the formed coupons and pins.
2. All of the tested Fe alloy materials have the potential to cause significant cytotoxicity in a direct contact *in vitro* environment. No viable cells from either cell line were seen to grow on any of the Fe alloy coupons. These results add further evidence to the already available literature, most of which is performed via indirect contact techniques.
3. The use of direct contact cytotoxicity testing more closely mimics an *in vivo* environment and was the technique of choice in this work. More recent literature is beginning to favour this technique over indirect methods of *in vitro* cytotoxicity testing.
4. Pre-corroding coupons for three days prior to direct contact *in vitro* cytotoxicity had no effect on the outcome ($P=0.999$) with all cells expiring within one day of seeding. While corrosion rate of the coupons was expected to slow over time, it had not

slowed sufficiently to allow the resultant cytotoxicity to be acceptable to the seeded cells.

5. Despite the usefulness of *in vitro* cytotoxicity testing, it is impossible to replicate an *in vivo* environment with complete accuracy. This is the major contributing factor to the differences seen between *in vitro* and *in vivo* results in this thesis. The dynamicity afforded by a living organism has the potential to replenish depleted bone-forming cells as well as wash away any toxic corrosion products.
6. The test Fe alloy materials are as yet not fit for use in a clinical situation. While *in vivo* biocompatibility is good, their corrosion rate and porosity are still not controllable to a degree which would allow them to function correctly as corroding bone graft substitutes.
7. The insoluble corrosion products formed in an *in vivo* situation hinder the corrosion rate of the implant. These products have the tendency to remain around the corroding implant, trapped by surrounding bone and soft tissues. This creates a physical barrier between uncorroded material beneath and the surrounding environment, improving cytotoxicity but slowing corrosion rate.
8. In order to remedy the slow corrosion rate of these materials, porosity and pore interconnectivity of the resultant implants need to be increased. This will aid the removal of insoluble corrosion products and the growth of bone through the

implant. This may however increase cytotoxicity and so a balance between these two is of utmost importance.

9. It is still unclear whether the addition of Ag increases the corrosion rate of Fe alloy materials. From the corrosion layer thickness of the explanted pins, it appears that a higher Ag content may increase the amount of corrosion product formed. Still, there was little overall corrosion within the six-month implantation period and so it can be concluded that the addition of Ag did not increase corrosion rate sufficiently.
10. The eventual Fe alloy clinical implant will need to find a delicate balance between cytotoxicity, corrosion rate and mechanical stability. Only then will they function correctly as corroding bone graft substitutes. It is not yet known whether Fe alloys will be able to offer this critical balance.

Recommendations and Future Work

There are still several deficiencies with regards to the ability of Fe-based alloys to fulfil their function as bioresorbable bone graft substitutes. In order to be able to provide a clearer answer to the questions asked, the following recommendations are being made:

1. Fe-based alloys have the potential to cause significant cytotoxicity *in vitro*. This however, is dependent on several factors such as the process employed to produce the scaffold, its resultant porosity and the methods (direct or indirect) used to test cytotoxicity. This has created a lot of heterogeneity in both testing methods and consequently, results that are available in the literature. The provision of clearer and more standardised guidelines regarding methods of testing, including recommendations on the specific cell lines to be used will allow for more homogenous methods and will yield more reliable and replicable results.
2. Corrosion of Fe alloy materials is a dynamic process. As such, the modification of *in vitro* testing techniques in the future may also help to increase their likeness to an *in vivo* situation. The changing of media on a regular basis (in direct contact cytotoxicity techniques) is one method by which this may be improved.
3. From the results of this thesis and those available in the literature, Fe-based alloys have so far proven to corrode at rates which are too slow for them to be effective bioresorbable bone graft substitutes. Future work will need to focus on several parameters including the methods of production of the scaffold, as this has

consequences on the final structure, porosity, corrosion rate and cytotoxicity of the implant.

4. One of the problems with the current alloys appears to be the passivating effect of corrosion products forming on their surfaces, slowing down their corrosion rates. A potential solution to this issue may be found through the addition of different alloying elements which may increase the solubility of the formed corrosion products. While this may increase the corrosion rate of the scaffolds produced however, it may also increase systemic toxicity through deposition at sites such as the liver, spleen, kidney and heart, decreasing biocompatibility.
5. *In vivo* tests are severely lacking. Most of the current evidence body focuses on *in vitro* techniques but as seen from the literature and from this thesis, these are limited in their ability to reproduce *in vivo* results. Materials which are apparently cytotoxic *in vitro* have had little to no ill-effect *in vivo* and so far, there has not been any exception to this.

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Appendices

Appendix 1 – Lists of Reagents, Materials and Equipment for *In Vitro* and *In*

Vivo Tests

Table 9(b) – Reagents used during the performance of cell culture techniques and *in vitro* cytotoxicity tests – Continued on four pages.

<u>Reagent</u>	<u>Brand</u>	<u>Function</u>
Completed hFOB medium	Prepared fresh	Completed hFOB medium containing 10% Foetal bovine serum (FBS), 0.3mg/ml G418 and DMEM-F12. Prepared fresh as needed in 200ml batches and kept for no longer than one month at 4°C. Completed medium was used for cell culture including to neutralise trypsin used during cell subculture techniques and during cytotoxicity testing
Completed MC3T3-E1 medium	Prepared Fresh	Completed MC3T3-E1 medium containing 10% FBS, 50 I.U./ml Penicillin, 50µg/ml Streptomycin and MEM α. Prepared fresh as needed in 200ml batches and kept for no longer than one month at 4°C. Completed medium was used for cell culture including to neutralise trypsin in cell subculture techniques and during cytotoxicity testing
Dulbecco's modified eagle medium/nutrient F12 (DMEM-F12)	Gibco™, Thermo Fisher Scientific, USA	Synthetic cell culture medium containing vitamins, salts, amino acids and carbohydrates as necessary to culture hFOB 1.19 cells (ATCC®, CRL-11372™). Importantly, the utilised medium did not contain phenol red indicator as per ATCC® specification

Table 9(b) – Reagents used during the performance of cell culture techniques and *in vitro* cytotoxicity tests
– Continued on four pages.

Foetal bovine serum (FBS)	Gibco™, Thermo Fisher Scientific, USA	Added to both DMEM-F12 and MEM α as part of completed medium. Contains proteins and growth factors which aid <i>in vitro</i> cell growth. FBS is able to inactivate trypsin enzyme through the action of α 1-antitrypsin
G418 disulfate salt solution (50mg/ml)	Sigma-Aldrich®, USA	An aminoglycoside antibiotic which blocks polypeptide synthesis in both prokaryotic and eukaryotic cells not having the Neomycin resistance gene (neo). The hFOB cell line for which this antibiotic was used is conditionally immortalised through transfection with a temperature sensitive mutant gene, along with the neo gene
Minimum essential medium α (MEM α)	Gibco™, Thermo Fisher Scientific, USA	Synthetic cell culture medium containing amino acids, carbohydrates and vitamins as necessary for culture of MC3T3-E1 cells. Contains phenol red indicator but not Ascorbic acid, as per ATCC® specification
Penicillin-Streptomycin (5,000U/ml)	Gibco™, Thermo Fisher Scientific, USA	An antibiotic containing Penicillin and Streptomycin which act on bacterial cell walls and ribosomes (interfering with protein synthesis) respectively. It is therefore effective against both gram-positive and gram-negative bacteria
Phosphate buffered saline (PBS)	Sigma-Aldrich®, USA	A powder reagent added to de-ionised water to form a 0.01M concentration of PBS, pH 7.4. It is a buffer solution containing salts which prevent the osmotic rupture of cells and is frequently used in cell culture techniques in order to maintain optimum pH conditions. It is also used as an additive to other solutions

Table 9(b) – Reagents used during the performance of cell culture techniques and *in vitro* cytotoxicity tests
– Continued on four pages.

CellTiter-Glo® luminescent cell viability assay	Promega, USA	A cell viability assay producing a luminescent signal based on the amount of ATP in the tested sample. Rapidly produces a signal which remains stable over several hours
Trypan blue solution (0.4%)	Invitrogen™, Thermo Fisher Scientific, USA	A cell staining solution used for cell counting techniques. Viable cells are able to select the compounds which cross their cells membrane and do not take this dye up. It is however taken up by non-viable cells and therefore stains these blue, allowing the counting of viable and non-viable cells
Trypsin (2.5%), without phenol red	Gibco™, Thermo Fisher Scientific, USA	A proteinase enzyme acquired at 10x strength and diluted with PBS, to 1x in this case. This enzyme is used during culture techniques of adherent cell lines to detach cells from each other and from the flask's culture surface, releasing them into suspension
Deionised water		Deionised water was used to dilute other solutions such as ethanol to the required strength while ensuring the maintenance of chemical purity
Ethanol	Gibco™, Thermo Fisher Scientific, USA	70% ethanol in water was used to sterilise all equipment entering the cell culture hood as well as the test metal coupons and pins for <i>in vitro</i> and <i>in vivo</i> tests. Dilutions ranging from 25-100% were used to fix cells on the coupons' surfaces before imaging with reflective light microscopy and SEM
Liquid nitrogen	Multigas, Malta	Liquid nitrogen has a boiling point of -195.8°C and was used to store cell lines over long periods of time

Table 9(b) – Reagents used during the performance of cell culture techniques and *in vitro* cytotoxicity tests
– Continued on four pages.

Fe alloy coupons		Pure Fe, FeMn, FeMn1Ag and FeMn5Ag materials manufactured via powder metallurgy and used for <i>in vitro</i> cytotoxicity testing
Stainless Steel 316LVM (SS316LVM)	Klein, Switzerland	This stainless steel is used in medical-grade equipment including within implants such as plates and screws for fracture fixation. It is resistant to corrosion and was used in this case as a control material for <i>in vitro</i> cytotoxicity tests
Thermanox™	Nunc™, Thermo Fisher Scientific, USA	Formed of a proprietary polyester material and resistant to several chemicals such as ethanol, these coverslips are cell-culture treated and allow for the adherence and growth of cells on their surfaces
Glutaraldehyde (25%)	Acros Organics, USA	A chemical which effectively crosslinks protein molecules within cells and helps to prevent their lysis. Because of this function, it was used as part of the cell fixing protocol in order to be able to visualise cells under SEM
Hexamethyldisilazane	TCI, Japan	A chemical used during the final steps of the cell fixing protocol. This chemical aids in the final dehydrating process of samples destined to be examined under SEM
Sodium cacodylate trihydrate buffer pH 7.3 (0.1M and 0.2M)	Sigma-Aldrich®, USA	An organic arsenic compound used as a buffer in cell fixing protocols. It is able to buffer the strong acidity that may result from the fixing procedure

Table 10(b) – Materials used during the performance of cell culture techniques and *in vitro* cytotoxicity tests – Continued on two pages.

<u>Material</u>	<u>Brand</u>	<u>Function</u>
Conical centrifuge tubes	Corning®, USA	Used in 15ml and 50ml varieties, these tubes are used for centrifugation purposes during cell subculture and <i>in vitro</i> cytotoxicity procedures
Microcentrifuge tubes	Corning®, USA	Used to contain smaller volumes of chemicals, such as a 40µl mix of cell suspension and Trypan blue during cell counting
Cell culture flasks	Thermo Fisher Scientific, USA	Sterile cell culture flasks used in 25cm ² (T25) and 75cm ² (T75) varieties. Treated specifically on their cell culture surfaces to improve adherence of cell lines
Micropipettes	Gilson®, France	Micropipettes allow for the accurate measurement of small volumes of fluid, which may contain cells. This is imperative in the performance of cell culture techniques and cytotoxicity testing as inaccuracies when dealing with small volumes may result in large margins of error
Serological pipettes	VWR International, USA	Used in 2ml, 5ml, 10ml and 20ml varieties during all cell culture and cytotoxicity testing procedures to transfer liquids in a sterile manner.
Cryovials	Corning®, USA	Small volume (<3ml) vials used to contain cells to be stored in liquid nitrogen
6-well plates	Corning®, USA	Clear 6-well plates used as each well could accommodate the 21.8mm diameter metal coupons
96-well, opaque-walled microplates	Corning®, USA	Opaque-walled microplates used in cell viability tests with CellTiter-Glo® assay. The opaque walls were necessary to prevent erroneous readings coming from luminescence in adjacent wells

Table 10(b) – Materials used during the performance of cell culture techniques and *in vitro* cytotoxicity tests
– Continued on two pages.

Cell counting slides	Invitrogen™, Thermo Fisher Scientific, USA	Cell counting slides used with the Countess™ 3 automated cell counter. Used for automated cell counting once staining with Trypan blue had been performed
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Table 11(b) – Equipment used during the performance of cell culture techniques and *in vitro* cytotoxicity tests – Continued on two pages.

Incubator	CellMate® CO ₂ Incubator – CO ₂ control, ESCO® Lifesciences, Singapore	Cell culture flasks as well as test metal coupons seeded with cells were placed here to allow cell growth. Allows regulation of humidity with Carbon dioxide (CO ₂) set to 5% and temperature set to 34°C for the hFOB cell line and 37°C for the MC3T3-E1 cell line, providing optimal cell growth conditions
Laminar flow cabinet	SafeFAST Elite, FASTER, DASIT Group, Italy	Laminar flow cabinet used for all cell handling and culture techniques, including cytotoxicity tests. The cabinet was cleaned with 70% ethanol and UV sterilised before and after use. All equipment, materials and reagents brought inside were also cleaned with the same ethanol solution
Automated cell counter	Countess™ 3 Automated Cell Counter, Invitrogen™, Thermo Fisher Scientific, USA	Employs Trypan blue mixed with a cell suspension to count cells. This piece of equipment must be calibrated to each cell line but is then faster than manual counting. It must be used with appropriate slides which are provided by the same manufacturer
Centrifuge	Centrifuge 5810, Eppendorf®, Germany	Rotatory device allowing for the separation and sedimentation of a conical centrifuge tube's contents based on density
Inverted light microscope	EVOS™ XL, Thermo Fisher Scientific, USA	Microscope with several objective lenses. Useful for observation of cell cultures including to ensure growth, the absence of infection and also for cell counting. Has a light source above and objective lenses below to be able to view adherent cells growing on the base of a cell culture flask

Table 11(b) – Equipment used during the performance of cell culture techniques and *in vitro* cytotoxicity tests – Continued on two pages.

Multimode microplate reader	Spark®, TECAN, Switzerland	Microplate reader with the ability to read fluorescence signals emitted during the performance of a CellTiter-Glo® cell viability assay
Orbital plate shaker	Eppendorf®, USA	Programmable plate shaker with settings for temperature, oscillations per minute and time. This piece of equipment was used to shake plates after the addition of CellTiter-Glo® Reagent, aiding cell lysis
Autoclave	Boxer, UK	Moist heat sterilisation system. Uses steam to generate heat and pressure in order to sterilise laboratory equipment
Fume hood	Industrial Laborum Ibérica, Portugal	Ventilated enclosure within which hazardous chemicals were handled, preventing injury to the user
Gold sputter-coater	Automatic Sputter Coater, Agar, USA	Automated gold sputter-coating system which allows for accurate SEM imaging of coupons and pins in <i>in vitro</i> and <i>in vivo</i> experiments respectively
Scanning Electron Microscope	FE-SEM, Merlin Gemini II column, Carl Zeiss AG, Germany	Scanning electron microscope used for the imaging of coupons and pins. EDS analysis was also made possible using an AMETEK® EDAX detector (USA)
Liquid nitrogen storage	Nitrogen Tank, Taylor Wharton, USA	Storage container for liquid nitrogen. Allows the storage of cells over long periods of time

Table 14(b) – Reagents used during *in vivo* biocompatibility tests.

<u>Reagent</u>	<u>Brand</u>	<u>Function</u>
Fe alloy pins		FeMn, FeMn1Ag and FeMn5Ag pins manufactured via powder metallurgy. Used for <i>in vivo</i> biocompatibility tests
Ethanol	Gibco™, Thermo Fisher Scientific, USA	70% ethanol in water was used to sterilise all equipment entering the cell culture hood as well as the test metal coupons and pins for <i>in vitro</i> and <i>in vivo</i> tests. Dilutions ranging from 25-100% were used to fix cells on the coupons' surfaces before imaging with reflective light microscopy and SEM
Isoflurane	Sigma Aldrich®, USA	Volatile anaesthetic agent which allows for the rapid induction of, and recovery from anaesthesia. Can be delivered in a controlled manner through a mask or within a chamber
Chloramphenicol 1% w/w ointment	URSAPHARM, Germany	An antibiotic ointment which inhibits protein synthesis. It is applied topically to sutured surgical wounds in order to help prevent infection in the early post-operative period
Formaldehyde	Sigma Aldrich®, USA	A chemical produced through the oxidation of methanol and used to fix tissue and bone specimens for histological examination
Formic acid	Sigma Aldrich®, USA	Weak acid used in the decalcification process of bone prior to its sectioning and histological staining. It is less likely to interfere with cells' nuclear structures than stronger acids

Table 15(b) – Materials used during *in vivo* biocompatibility tests.

<u>Material</u>	<u>Brand</u>	<u>Function</u>
Sutures	Polypropylene 4/0, SMI, Belgium	Monofilament, non-absorbable suture which is ideal for the closure of wounds. Its monofilament structure decreases the risk of infection

Table 16(b) – Equipment used during *in vivo* biocompatibility tests – Continued on two pages.

<u>Equipment</u>	<u>Brand</u>	<u>Function</u>
Anaesthetic chamber	Sliding top chamber, Kent Scientific®, UK	Sliding top chamber in which a rat is placed. Allows delivery of a volatile anaesthetic agent to achieve rapid and painless anaesthesia
Anaesthetic delivery system	SomnoSuite®, Kent Scientific®, UK	Delivery system for volatile anaesthetic agents such as Isoflurane. Allows for the consistent and controlled delivery of these agents to induce and maintain an adequate depth of anaesthesia
Anaesthetic mask	Low-profile anaesthesia mask, Kent Scientific®, UK	Close-fitting mask delivering volatile anaesthetic agents to anaesthetised animals for the maintenance of anaesthesia
Surgical pad	SurgiSuite®, Kent Scientific®, UK	Heated surgical pad with magnifying glass and lighting attachments. Provides a controlled area where equipment and surgical site can be kept clean. Needs to be heated as rats lose their ability to accurately thermoregulate whilst anaesthetised
Surgical Instruments	Kent Scientific®, UK	A number of surgical instruments were required to perform <i>in vivo</i> biocompatibility tests, including needle holders, forceps, scissors and blades. These were sterilised prior to being used

Table 16(b) – Equipment used during *in vivo* biocompatibility tests – Continued on two pages.

Microdrill	OmniDrill35, World Precision Instruments, USA	Microdrill with several attachments of different size. Allows the creation of defects within bone of consistent size
Histological microscope	AxioStar Plus Binocular Microscope, Carl Zeiss AG, Germany	Microscope with several objective lenses and ideally equipped with a camera in order to be able to capture images of the histological specimens being examined
Manual rotary microtome	RM2235, Leica, USA	Microtome with the ability to section fixed histological specimens in thicknesses varying from 1-60µm
Tissue Processor	Vacuum Tissue Processor MTM-II, SLEE, Germany	Automated tissue processor used for the fixation of histological specimens within paraffin in preparation of histological examination
Gold sputter-coater	Automatic Sputter Coater, Agar, USA	Automated gold sputter-coating system which allows for accurate SEM imaging of coupons and pins in <i>in vitro</i> and <i>in vivo</i> experiments respectively
Scanning Electron Microscope	FE-SEM, Merlin Gemini II column, Carl Zeiss AG, Germany	Scanning electron microscope used for the imaging of coupons and pins. EDS analysis was also made possible using an AMETEK® EDAX detector (USA)

Appendix 2 – Ethical Approval for *In Vivo* Experimentation



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Ref No: FRECMDS_2021_060

8 March 2021

Luke Saliba
44, 'Green-Tangle',
Godwin Ganado Street,
Tal-Virtu
Rabat

Dear Mr Saliba,

With reference to your application submitted to the Faculty Research Ethics Committee in connection with your research entitled:

Biodegradable FeMn Bone Graft Substitutes: In vitro and in vivo testing

The Faculty Research Ethics Committee granted ethical approval for the above mentioned application reviewed on 24 February 2021, following the recommendation by the Joint FREC Animal Research Sectoral Subcommittee (JFARSS).

Yours sincerely,

A handwritten signature in blue ink, appearing to read 'Pierre Mallia'.

Professor Pierre Mallia
Chairman
Faculty Research Ethics Committee

Appendix 3 – Supplementary EDS Results showing Atomic Percentages

Table 17(b) – EDS analyses of the surface (1) and pores (2) of a pure Fe coupon showing the atomic percentages of the detected elements.

Element	Fe	O	C	Mn	Mg	Al	Si	Ca	Ti	V
%At – 1	70.68	7.26	22.06	-	-	-	-	-	-	-
%At – 2	1.57	62.93	7.40	0.88	1.77	7.09	10.73	3.32	3.40	0.50

Table 19(b) – EDS analyses of the surface (1) and pores (2) of an FeMn coupon showing the weight percentages of the detected elements.

Element	Fe	Mn	O	C	N	Mg	Al
%At – 1	42.02	22.73	5.08	18.22	11.96	-	-
%At – 2	-	29.51	57.93	10.13	-	00.67	1.75

Table 21(b) – EDS analyses of the surfaces (1, 4), pores (2, 5) and bright areas (3, 6) of FeMn1Ag (1-3) and FeMn5Ag (4-6) coupons, showing the atomic percentages of the detected elements.

Element	Fe	Mn	Ag	O	C	N	Al	Si	Mg
%At – 1	42.81	22.79	-	5.04	17.82	-	-	-	-
%At – 2	-	31.11	-	56.97	9.74	-	1.48	-	0.70
%At – 3	2.07	5.07	31.17	16.58	36.90	7.17	-	0.26	0.79
%At – 4	37.15	18.85	-	4.45	29.37	10.18	-	-	-
%At – 5	-	17.78	-	40.44	41.78	-	-	-	-
%At – 6	-	3.73	18.05	7.01	7081	-	-	-	0.39

Table 23(b) – EDS analyses of the surface (1) and pores (2) of a pure Fe coupon after the acid etching procedure, showing the atomic percentages of the detected elements.

Element	Fe	O	C	Mn	Mg	Al	Si	Ca	Ti	V
%At – 1	22.09	9.65	22.09	-	-	-	-	-	-	-
%At – 2	1.48	66.16	-	3.16	6.24	14.73	1.77	0.30	5.07	1.07

Table 25(b) – EDS analyses of the surface (1) and pores (2) of an FeMn coupon after the acid etching procedure, showing the atomic percentages of the detected elements.

Element	Fe	Mn	O	C	N	Cl
%At – 1	38.14	21.23	12.35	16.74	10.47	1.08
%At – 2	40.34	22.29	14.74	13.45	8.26	0.92

Table 27(b) – EDS analyses of the surfaces (1, 4), pores (2, 5) and bright areas (3, 6) of FeMn1Ag (1-3) and FeMn5Ag (4-6) coupons, showing the atomic percentages of the detected elements.

Element	Fe	Mn	Ag	O	C	N	Cl	Si	Mg
%At – 1	34.80	20.12	-	16.71	18.63	9.15	0.34	0.24	-
%At – 2	35.61	20.19	-	16.25	17.23	8.58	2.15	-	-
%At – 3	3.05	5.88	17.04	46.07	22.27	4.87	0.32	-	0.50
%At – 4	42.82	20.64	-	5.95	19.33	10.88	-	0.36	-
%At – 5	29.40	15.04		3.84	43.28	8.44			
%At – 6	4.02	4.87	32.38	18.82	39.07	-	-	-	0.95

Table 35(b) – EDS analyses performed on areas of uniform (1), and seemingly increased (2) corrosion on the surface of a pure Fe coupon after four days of *in vitro* cytotoxicity testing. Atomic percentages of the detected elements are shown.

Element	Fe	O	C	N	Na	Mg	Al	Si	P	Ca
%At – 1	60.74	14.66	17.83	5.69	1.09	-	-	-	-	-
%At – 2	20.99	43.10	23.70	6.98	2.60	0.45	0.10	0.15	1.70	0.24

Table 37(b) – EDS analyses performed on areas of uniform (1) corrosion as well as corrosion products (2) present over the majority of the surface of an FeMn coupon. Areas of seemingly increased (3) corrosion have also been analysed with atomic percentages of the detected elements being shown.

Element	Fe	Mn	O	C	N	Na	Al	Si	P	Ca
%At – 1	42.79	16.23	14.62	18.16	6.01	0.72	0.28	0.27	0.74	0.18
%At – 2	20.18	5.10	42.30	19.87	5.61	2.68	0.08	0.25	3.38	0.55
%At – 3	20.82	2.69	44.59	19.76	5.77	2.83	0.07	0.42	2.51	0.54

Table 39(b) – EDS analyses of the surfaces of FeMn1Ag (1) and FeMn5Ag (2) coupons showing the atomic percentages of the detected elements.

Element	Fe	Mn	Ag	O	C	N	Na	Mg	Si	P	Ca	Al
%At – 1	28.32	16.20	0.07	24.17	22.74	5.65	1.52	0.28	0.24	0.50	0.30	-
%At – 2	23.74	1.69	0.00	47.35	20.06	4.86	0.88	0.18	0.28	0.66	0.22	0.07

Table 41(b) – EDS analyses of the acellular (1) and cellular (2) regions of an SS316LVM coupon showing the atomic percentages of the detected elements.

Element	Fe	Mn	Ni	Cr	Mo	O	C	N	Na	Cl	P
%At – 1	34.69	1.08	7.88	10.92	1.05	0.00	34.66	8.47	-	0.10	0.21
%At – 2	8.39	0.28	1.65	2.91	0.47	7.63	65.96	11.07	0.28	0.42	0.60

Table 44(b) – EDS analyses of the explanted FeMn (1, 2), FeMn1Ag (3, 4) and FeMn5Ag (5, 6) pins with 1, 3 and 5 denoting the superficial layers and 2, 4 and 6 denoting the deeper layers. The atomic percentages of the detected elements are shown.

Element	Fe	Mn	O	C	N	Ca	P	Na	S	K	Ag
%At – 1	6.85	0.76	50.97	27.43	5.35	1.73	5.27	0.94	0.19	0.51	-
%At – 2	5.83	2.62	39.80	41.76	7.78	0.25	0.78	0.32	0.87	-	-
%At – 3	3.91	0.76	46.27	35.60	8.11	0.71	2.98	0.86	0.19	0.39	-
%At – 4	2.89	1.60	38.80	47.96	6.34	0.53	1.17	0.23	0.22	0.05	-
%At – 5	7.27	0.53	48.17	29.72	5.80	1.48	4.90	0.91	0.34	0.72	-
%At – 6	7.02	3.32	53.55	27.95	6.29	0.07	0.79	0.17	0.47	0.06	-

Table 46(b) – EDS analyses of cross-sections of the explanted FeMn (1), FeMn1Ag (2) and FeMn5Ag (3, 4) pins. The atomic percentages of the detected elements are shown.

Element	Fe	Mn	O	C	Ca	P	K	Na	Ag
%At – 1	8.28	2.00	36.09	47.91	0.59	4.41	0.29	0.44	-
%At – 2	8.90	1.83	35.25	47.44	1.39	4.67	0.53	-	-
%At – 3	5.91	0.41	31.28	57.89	0.88	2.53	0.46	0.63	-
%At – 4	10.14	0.50	32.96	54.64	0.15	1.17	0.14	0.30	-