DESIGN, DEVELOPMENT, AND VALIDATION OF SPECIFIC METHODS TO ASSESS THE BIOLOGICAL PERFORMANCES OF DEGRADABLE METALS FOR CARDIOVASCULAR STENTS

Thèse

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Résumé

Les métaux biodégradables (MBs) ont été développés pour des applications spécifiques dans les domaines de l'orthopédie, la pédiatrie et le système cardiovasculaire. Les MBs sont conçus pour servir de support temporaire et une fois que leur présence n'est plus nécessaire, ils devront disparaitre progressivement grâce au processus de corrosion. Ces dernières décennies, divers tests aussi bien *in vitro* que *in vivo* ont été effectués dans le but de comprendre le comportement à la corrosion des biomatériaux métalliques conçus pour être résistants à la corrosion. Les MBs forment une classe de matériaux relativement nouveau et donc rares sont les tests effectués dans le domaine. Les tests effectués pour améliorer la résistance à la corrosion des biomatériaux métalliques ne peuvent pas non plus être simplement transposés aux MBs. Dans certains cas, ils peuvent être adaptés avec quelques modifications tandis que dans d'autres, la dégradation progressive devra être prise en compte pour la conception et à la mise au point de tests spécifiques. Le défi actuel est de savoir comment évaluer la réaction des tissus environnants et des organes en présence des produits de dégradation. Dans le cadre de ce projet, nous avons exploré une nouvelle méthode qui permet d'établir le profil d'expression des gènes (PEG) des cellules fibroblastique 3T3 de souris exposées à l'alliage Fe-35Mn. Cet alliage de fer récemment développé comme matériau métallique dégradable a été utilisé dans le cadre de cette expérience pour mieux comprendre le comportement des cellules face à des MBs potentiellement cytotoxiques. En résumé, les cellules 3T3 ont étés exposées pendant 24h aux élutions de produits de dégradation à travers un filtre de culture cellulaire contenant des quantités cytostatiques de 3.25mg/ml pour Fe-35Mn en poudre, 0.25mg/ml de poudre de Mn pur ou 5mg/ml de poudre de fer pur. Le profil de l'expression des gènes a été établi pour ces cellules. En comparant l'expression du profil des gènes des cellules fibroblastes 3T3 en présence de Fe-35Mn et Mn, nous avons observé que l'expression de 68 gènes avait été augmentée et l'expression de 54 gènes abaissée. Nous avons testé chez 11 gènes modulés dans les « microarrays » si cette régulation était toujours présente en utilisant le RT-PCR quantitative. C'était le cas pour 10 d'entre eux. Nous avons constaté que la caveoline-1 (cav1), une protéine structurale de caveoles (invaginations lisses de la membrane plasmique), est l'un des gènes les plus régulés de notre GEPs. Nous avons par ailleurs étudié le potentiel d'utiliser cette protéine de 22KDa comme biomarqueur pour l'étude de la cytotoxicité lors de l'exposition aux MBs. Dans le but de mieux caractériser l'expression de la cav1 dans ce contexte, les cellules 3T3 ont étés exposés soit aux ions fer ou manganèse à des concentrations cytostatiques pendant 24h et 48h. L'expression de la cav1 n'a pas été influencée par l'exposition aux ions fer. Par contre l'exposition aux ions manganèse réduit l'expression des gènes de cav1 de 30% pendant 24h et de plus de 65% pendant 48h comparée au control. La contenu en protéin de la cav1 a également été réduit de façon semblable Le même phénomène a été observé pour la cav3 (le sous-type musculaire de la caveoline) dans cette étude. Cette tendance forte et reproductible de la régulation des cavéolines permet ainsi de le considérer comme un biomarqueur potentiel pour les MBs. Ce type de réponse de la caveoline à l'exposition des MBs est similaire chez les cellules endothéliales, fibroblastes et musculaires.

Summary

Biodegradable metals (BMs) have been introduced and proposed for some specific applications including cardiovascular applications. BMs are expected to undergo 'auto-withdrawal' through corrosion process after fulfilling structural support. In the past decades, a wide and complete set of *in vitro* and *in vivo* tests have been proposed and investigated for the conventional corrosion resistant metallic biomaterials--but not for the BMs. The same tests that the ones developed for corrosion-resistant metals cannot be simply transposed. They can be adapted in some cases while in some others progressive degradation should inspire and lead to the design and the development of new specific tests. The current challenge is how to assess the tolerance of surrounding tissues to the presence of degradation products. In this doctoral project, a new method to investigate BMs was explored by establishing the gene expression profile (GEP) of mouse 3T3 fibroblasts exposed to Fe-35Mn, recently developed iron-based alloy, in order to better understand cell response to potentially cytotoxic BMs. Briefly, 3T3 cells were exposed to degradation products eluting through tissue culture insert filter containing cytostatic amounts of 3.25 mg/ml of Fe-35Mn powder, 0,25 mg/ml of pure Mn powder or 5 mg/ml of pure iron powder for 24 hours. GEP was then conducted from these cells. When comparing the GEP of 3T3 fibroblasts in presence of Fe-35Mn and Mn, 68 up-regulated and 54 down-regulated genes were common. These results were confirmed by quantitative RT-PCR for a subset of these genes. It was found that caveolin-1 (cav1), the structural protein of caveolae little plasma membrane smooth invaginations present in various differentiated cell types, was one of the most down-regulated genes in our GEPs. We further studied the potential of this 22kDa protein to become a biomarker for cytotoxicity towards the exposure of BMs. In order to better characterize cav1 expression in this context, 3T3 mouse fibroblasts were exposed to either ferrous and manganese ions at a cytostatic concentrations for 24 or 48 hours. Cav1 gene expression was not influenced by exposition to ferrous ions. On the other hand, manganese exposure for 24 hours reduced cav1 gene expression by about 30% and >65% at 48 hours compared to control 3T3 cells. Cav1 cellular protein content was also reduced to the same extent. The same pattern of expression for cav3 (the muscle specific caveolin subtype) was also observed in the study. This strong and reproducible pattern of regulation of caveolins thus exhibits a potential as a biomarker for the toxicity of BMs.

Table of content

Chapter 3

Assessing the biocompatibility of degradable metallic materials: State of the art and focus on the genetic regulation potential

Chapter 4

Gene expression profile of mouse fibroblasts exposed to a biodegradable iron alloy for stents

Chapter 5

Caveolin: a possible biomarker of degradable metallic materials toxicity on vascular cells

Chapter 6

Discussion

List of tables

List of figures

List of abbreviation and medical glossary

Angiography: the radiographic visualization of the blood vessels after injection of a radiopaque substance

Angioplasty: surgical repair or recanalization of a blood vessel

Arterial Remodelling: changes in vascular dimensions during the development of atherosclerosis

Artery: any of the tubular branching muscular- and elastic-walled vessels that carry blood from the heart through the body

ASTM: American society for testing and materials

Atheroma: an abnormal fatty deposit in an artery

Atherosclerosis: an arteriosclerosis characterized by lipid deposits in and fibrosis of the inner layer of the arteries

Biocompatibility: the condition of being compatible with living tissue or a living system by not being toxic or injurious and not causing immunological rejection

Biodegradable: capable of being broken down especially into innocuous products by the action of living things (as microorganisms)

BMS: Biodegradable metal stent

Carcinogen: a substance or agent causing cancer

Catheter: a tubular medical device for insertion into canals, vessels, passageways, or body cavities for diagnostic or therapeutic purposes (as to permit injection or withdrawal of fluids or to keep a passage open)

CAD: Coronary artery disease

Cav: Caveolin

Col: Collagen

CRMs: Corrosion resistant materials

CVD: Cardiovascular disease

DNA: Deoxyribonucleic acid

ECs: Endothelial cells

ECM: Extracellular matrix

Enzyme: any of numerous complex proteins that are produced by living cells and catalyze specific biochemical reactions at body temperatures

Infarction: the process of forming an area of necrosis in a tissue or organ resulting from obstruction of the local circulation by a thrombus or embolus

Intima: the innermost coat of an organ (as a blood vessel) consisting usually of an endothelial layer backed by connective tissue and elastic tissue - called also tunica intima

Ischemia: deficient supply of blood to a body part (as the heart or brain) that is due to obstruction of the inflow of arterial blood (as by the narrowing of arteries by spasm or disease)

ISO: International standard organization

Lumen: the cavity of a tubular organ (the lumen of a blood vessel)

MMP: Matrix metalloproteinase

Necrosis: death of a portion of tissue differentially affected by local injury

Neo-intimal: a new or thickened layer of arterial intima formed especially on a prosthesis or in atherosclerosis by migration and proliferation of cells from the media

Patency: the quality or state of being open or unobstructed (evaluating arterial patency)

Plaque: an atherosclerotic lesion

Platelet: blood cell fragments that are involved in the cellular mechanisms that lead to the formation of blood clots

PCR: Polymerase chain reaction

Proliferation: rapid and repeated production of new parts or of offspring (as in a mass of cells by a rapid succession of cell divisions)

Pulmonary: relating to, functioning like, associated with, or carried on by the lungs

Restenosis: the reoccurrence of stenosis in a blood vessel or heart valve after it has been treated (as by balloon angioplasty or valvuloplasty) with apparent success

Revascularization: a surgical procedure for the provision of a new, augmented, or restored blood supply to a body part or organ

RNA: Ribonucleic acid

SMCs: Smooth muscle cells

Sirolimus: a relatively new immunosuppressant drug used to prevent rejection in organ transplantation, and is especially useful in kidney transplants. It is also known as rapamycin.

Stenosis: A narrowing or constriction of the diameter of a bodily passage or orifice

Stent: An expandable wire meshes or hollow perforated tube that is inserted into a hollow structure of the body to keep it open

Stenting: A surgical procedure or operation for inserting a stent into an anatomical vessel

Strut: A structural component designed to resist longitudinal compression

Thrombogenicity: Tending to produce a thrombus

Thrombosis: The formation or presence of a blood clot within a blood vessel

Thrombus: A clot of blood formed within a blood vessel and remaining attached to its place of origin

TGF: Tumor growth factor

TNF: Tumor necrosis factor

Sources:
Medical dictionary, Merriam Webster:
http://www.nlm.nih.gov/medlineplus/mplusdictionary.html, Accessed on June 2014.

Dedication

I dedicate this thesis to My Beloved Mom, Tia Djuwita.

You are humble, simple, and warm. You would not give a second thought of sharing what you have with others although you do not have enough for yourself. You have given me the best life lesson of all time.

I could not thank you enough for your support and your restless nights working to support my education.

Your love, your sacrifice, your dreams, your belief, and your values have taken me so high yet I am so far away from you.

Thank you Mom for accepting me the way I am and keeping my name in your prayers.

You are always in my heart Mom.

I also dedicate this thesis to my father Mulyanto and my sister Ajeng Kurnia. Thanks for your love and support through countless calls days and nights. Thanks for bringing joy and laughter in my life. I really miss you guys.

Milan, June 11th 2015

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Forewords

Biocompatibility refers to the ability of a material to perform a particular function within living tissues minimizing the host response. Nowadays, stainless steel is considered as a gold standard of biocompatibility for implant materials. Therefore, available standards to assess the biocompatibility are all directed to corrosion resistant materials. However, biodegradable metals (BMs) are now considered as a potential material for the fabrication of cardiovascular stent. Therefore, standards standard for biocompatibility assessment need to be redefined for this new emerging class of materials—BMs.

As the on-going discovery of new potential materials such as in the fabrication cardiovascular stents continues, there has been a limited concern in assessing the biocompatibility. We decided to address this in this PhD project. With the guidance of Prof. Diego Mantovani and Prof. Jacques Couët as my director and co-director respectively, I have tackled this challenge of defining new ways to assess biocompatibility of BMs.

This thesis was prepared as an article insertion thesis. It includes three published articles in which I have taken the role as the principle investigator as well as the first author. The first article is a review article entitled: Assessing the biocompatibility of degradable metals: state of the art and focus on the genetic regulation potential. The article was co-authored by Dr. Hendra Hermawan, Dr. Diego Mantovani, and Dr. Jacques Couët. It was published in the journal Acta Biomaterialia, 2010, Vol. 6, Issue 5, Page 1800-1807. My contribution to this article consists of designing the concept of the article, communicating ideas with co-authors, and preparing the first draft of the article. The co-authors revised the manuscript prior to submission.

The second article entitled: Gene expression profile of mouse fibroblasts exposed to a biodegradable iron alloy for stents. It was published in the journal Acta Biomaterialia, 2013, Volume 9, Issue 10, Page 8746-8753. The participating co-authors are Dr. Serge Champetier, Dr. Hendra Hermawan. Dr. Diego Mantovani, and Dr. Jacques Couët. The concept of the article was designed with the suggestion from Dr. Couët and Dr. Mantovani. My other contribution including experimental setup, cell culture, mRNA extraction and quantification, quantitative real time – polymerase chain reaction (RT-PCR) analysis, and preparation of the manuscript. Dr. Hermawan, a former graduate student at the same department, provided the samples and experimental setup, and Dr. Champetier, a former research professional, contributed in bioinformatics analysis regarding the DNA microanalysis data.

The third article entitled: Caveolin: a possible biomarker of degradable metallic materials toxicity on vascular cells. It was co-authored by Dr. Jacques Couët and Dr. Diego Mantovani. The article was published in the journal Acta Biomaterialia, 2013, Volume 9, Issue 10, Page 8754-8760. My contribution includes experimental setup, cell culturing, mRNA extraction, RT- PCR analysis, protein extraction, Western blotting, and manuscript preparation. Dr. Couët and Dr. Mantovani gave conceptual inputs and revision of the manuscript prior to submission.

Chapter 1

Introduction

1.1 Biomaterials and biocompatibility

The earliest report on material utilisation for medical purpose dates back 3000 B.C. in ancient Egyptian culture describing the practice of surgical suture. In the modern age, the same class of material was used more prominently during the World War II to treat wounded soldiers [1]. Since then, the concept of biomaterials has evolved considerably. In a general sense, a biomaterial is defined as any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual [2].

The early generation of biomaterials was designed to be inert hence providing only physical support with minimum requirement of compatibility. Nowadays, the concept of biomaterials has shifted significantly. Biomaterials are now seen as active substances, not only inert, that support the healing process of an injured tissue. They come in various forms such as a complex hybrid of composites or metals coated with different polymers [3]. Usually, a single material goes for a specific application. Various forms of biomaterials with their specific application are listed in Table 1.1.

Table 1.1. Available biomaterials and their applications. They are at least four classes of biomaterials consist of polymers, metals, ceramics, and composites. Each of the material has their own advantages and disadvantages, and they are applied for different applications of medical devices [2, 3].

Materials	Advantages	Disadvantages	Applications
Polymers Nylon, silicone rubber, polyester, polytetrafuoroethylene, etc.	Tailorable mechanical properties Easy to fabricate Biodegradable Surface modification Immobilize cells	Leachable compounds Absorps water, protein, etc. Surface contamination Wear & breakdown Difficult to sterilize	Sutures, blood vessels, hip socket, ear, nose, other soft tissues
Metals Ti alloys, Co-Cr alloys, SS, Au, Fe, Pt, etc.	High strength Fatigue resistance Wear resistance Easy fabrication Easy to sterilize Shape memory	High modulus Corrosion Metal ion sensitivity Metal ion toxicity	Join replacements, bone plates and screws, dental roots, pacer and suture wires
Ceramics Alumunium oxide, calcium phosphates, carbon	High compression Wear and corrosion resistance Bioactive/inert	High modulus Low fracture toughness Difficult to fabricate	Dental, femoral head of hip replacement, coating of dental and orthopaedic implants Difficult to make
Composites Carbon-carbon, wire or fiber reinforced bone cement	Strong, tailor-made	Difficult to make	Join implants, heart valves

In the early development of biomaterials, there was a limited concern about how a material would interact with human body since they were considered inert. However, as the demand for biomaterials kept increasing, the development of non-inert biomaterials added a new layer of complexity. Consequently, the need of a systematic study to assess the compatibility of various materials become important permitting a standardized assessment to compare new biomaterials with the previously existing ones. The term of biocompatibility was then introduced in the field. Biocompatibility refers to the ability of a material to perform a particular function within living tissues followed by appropriate host responses—minimum inflammatory and toxicity reactions both locally and systematically [2]. It is thus a complex function of materials, design, applications, host responses, etc.

1.2 A new emerging class of biomaterials: Biodegradable metals (BMs)

Biocompatibility is a compulsory requirement for implants including cardiovascular stent materials. Although stainless steel has long been considered the material of choice for the fabrication of stents, other materials have been proposed with equivalent mechanical properties and biocompatibility to those of stainless steel. Moreover, a novel class of material, biodegradable metals (BMs), was recently proposed as an alternative to permanent metallic implants [4]. One well-known application of BMs is biodegradable stent. Degradable stent could help avoid toxic element leakage leading to late inflammatory responses or secondary intervention due to perpetual stent diameter against growing artery in young patients. Guidelines for biocompatibility assessment have been issued by the International Standard Organization (ISO) as shown in Table 1.2, however they were not specifically developed for BMs.

Table 1.2. Standards of ISO 10993 series for biocompatibility assessment of medical devices. In most cases the standards are issued jointly with Comité Européen de Normalisation (CEN) [5].

ISO standard	Title
ISO 10993-1	Evaluation and testing
ISO 10993-2	Animal welfare requirements
ISO 10993-3	Tests for genotoxicity, carcinogenicity, and reproductive toxicity
ISO 10993-4	Selection of tests for interactions with blood
ISO 10993-5	Tests for in vitro toxicity
ISO 10993-6	Tests for local effects after implantation
ISO 10993-7	Ethylene oxide sterilization residuals
ISO 10993-9	Framework for identification and quantification of potential degradation products
ISO 10993-10	Tests for irritation and delayed-type hypersensitivity
ISO 10993-11	Tests for systemic toxicity
ISO 10993-12	Sample preparation and reference materials
ISO 10993-13	Identification and quantification of degradation products from polymeric devices
ISO 10993-14	Identification and quantification of degradation products from ceramic devices
ISO 10993-15	Identification and quantification of degradation products from metals and alloys
ISO 10993-16	Toxicokinetic study design for degradation products and leachables
ISO 10993-17	Establishment of allowable limits for leachable substances
ISO 10993-18	Chemical characterization of materials
ISO/TS 10993-19*	Physicochemical, morphological, and topographical characterization of materials
ISO /TS 10993-20*	Principles and methods for immunotoxicological testing of medical devices

**TS = Technical specification only, no ISO standard*

Development of new biomaterials needs to be accompanied with new effective tests to assess their biocompatibility. As new alloys are developed meeting mechanical standard and have the potential for implantation in patients, rapid screening procedures are needed in order to assess their biocompatibility. These screening tests will help identify candidate materials considered biologically compatible. Screening tests can act as a step in the development of new materials. Unfortunately, there is no appropriate method to assess the biocompatibility of the BMs for cardiovascular stent application [6].

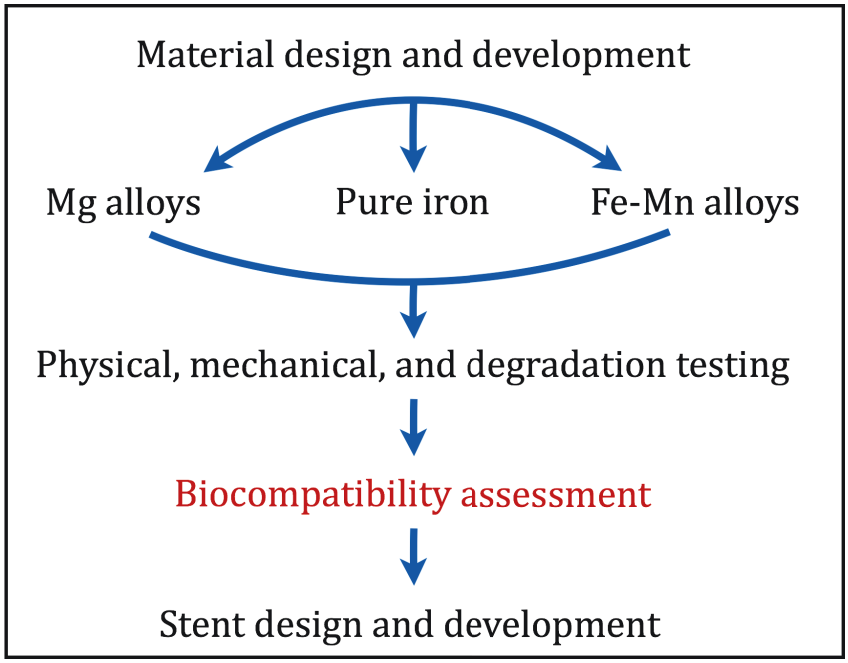


Figure.1.1. Flowchart of BMs (biodegradable metals) development for cardiovascular stent application. This doctoral project is part of BMs research team at the Laboratory of Biomaterials and Bioengineering (LBB), Laval University. The project is emphasized on the biocompatibility assessment of BMs as an important stage in material development.

The Laboratory of Biomaterials and Bioengineering of the Department of Metallurgy and Materials Engineering at Laval University, is one of the world leading research groups working on BMs for cardiovascular stent application. Previous publications from Levesque *et al.* [7]*;* Moravej *et al.* [8]*;* and Hermawan *et al.* [9] have showed the development of BMs for cardiovascular stent application focusing on magnesium alloy, pure iron and iron-based alloy respectively, as shown in Figure 1.1. Levesque *et al.* [7] studied the degradation behaviour of a magnesium alloy, AM60B-F as a BM for stent material. Moravej *et al.* [8] have developed pure iron fabricated by electrodeposition, while Hermawan *et al.* [9] have developed iron-manganese alloys with enhanced corrosion rate. It is important to supplement these contributions to the field with appropriate methods to assess their biocompatibility status. This doctoral project was focused on the development of new ways to assess BMs biocompatibility.

As shown in Table 1.2, some tests have already been used to screen permanent implant materials following ISO 10993 standards, but they are not specifically designed to assess BMs. Some questions that remain to be answered. 1) What are the responses of the diseased artery exposed to a stent made of BMs?; 2) Is there a safety risk from the degradation by-products of BMs to the recipient?; 3) How long is 'long enough' for the stent to remain in the diseased artery as a scaffold. Answers to those questions are needed in order (1) to help the development of degradable materials with minimal effect to the local physiology, (2) to identify potential biocompatibility markers and (3) to provide information about the time sufficient enough for the materials to be completely degraded within the artery. In summary, the development of degradable metallic materials is expected to match the biocompatibility requirements for degradable metallic stents.

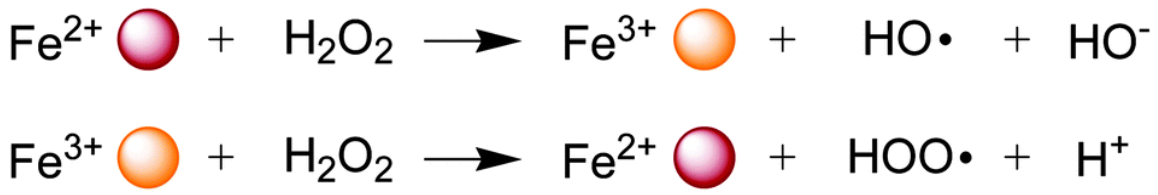


Figure 1.2. Fenton reaction. Iron (II) is oxidized by hydrogen peroxide to iron (III), forming a hydroxyl radical and a hydroxide ion in the process. Iron (III) is then reduced back to iron (II) by another molecule of hydrogen peroxide, forming a hydroperoxyl radical and a proton. The net effect is a disproportionation of hydrogen peroxide to create two different oxygen-radical species, with water ($H^+ + OH^-$) as a by-product.

1.3 New class of material requires a new biocompatibility approach

The requirements for biomaterials are evolving, thus the study of biocompatibility needs to be approached or adjusted accordingly. The earlier generation of biomaterials has emphasized the inert property avoiding the occurrence of degradation process through degradation, corrosion, hydrolysis, etc. For this reason, the biocompatibility assessment standards released by ISO were designed in favour to non-degradable materials and they are referred as the conventional assessment method. The conventional method is based on phenotypic measurements such as cell death, cell adhesion, cell proliferation, the presence of inflammatory cells, vascularisation, etc. [6].

Newer generations of biomaterials are expected to support the healing process, if not becoming a part of the surrounding tissues, and to favour the remodelling process of human body. Biodegradable metals are amongst the new generations of biomaterials. They are designed to provide a temporary scaffolding function and disappear gradually through corrosion process. Corrosion process releases degradation products mainly in the form of metallic ions that create imbalanced charge or oxidative stress within the cells and surroundings. Metallic ions are likely to participate in the Fenton reaction producing reactive oxygen species as seen in Figure 1.2. Reactive oxygen species are hazardous to the stability of the genetic code—DNA (Deoxyribonucleic acid). They are closely involved in DNA damage and alteration of genetic regulation resulting in cell death (toxicity) or cellular over proliferation (carcinogenicity) [10]. Since toxicity and carcinogenicity are attributed to the alteration of genetic regulation in the presence of degradation products, it is then important to study it as an approach towards biocompatibility evaluation. This become more appealing in the case of degradable metals since they release degradation products including free metallic ions.

Since the conventional assessment methods are dedicated for non-corrosive metals, they are not considering the constant release of metallic ions. A new approach to assess the biocompatibility of degradable metals has to consider the constant release of metallic ions affecting the genetic regulation of the surrounding tissues. For this reason, this doctoral project is emphasizing the study of genetic regulation as a new approach to assess the biocompatibility of biodegradable metals. This new approach is based on profiling gene expression modifications instead of evaluating phenotypic changes. However, genetic alteration is not always manifested phenotypically. Therefore, phenotypic measurement does not provide integrative information of degradation products towards cellular behaviours such as cell adhesion, proliferation, metabolism, cell death, etc. [6].

Since the new approach is based on gene expression measurement, more subtile disturbance caused by degradation products affecting surrounding cells can be evaluated. The application of this new approach involves the employment of gene markers or biomarkers. Biomarkers will act as a reporter towards the change of genetic regulation due to the presence of the degradation products. Therefore, the sensibility of the measurement is expected to be superior to that of phenotypic measurement applied in the conventional method.

For this reason, the project is focused on the potential of gene regulation study as a new approach to assess the biocompatibility of BMs. The project considers the role of fibroblast cells, as they constitute the structure of the artery together with smooth muscle cells and endothelial cells. Fibroblast cells play an important role in the production of extracellular matrix (ECM), growth factors, cytokines, etc. Additionally, fibroblast cells are convenient to use in culture allowing various experimental setups. Therefore, we expect to establish the genetic regulation study on fibroblast cells for the first step on the project. Subsequently, we expect to identify potential biomarkers that applicable in assessing the biocompatibility of BMs.

1.4 Objectives of the project

The general objective of the project is to design, develop and validate new methods to assess the biocompatibility of biodegradable metals (BMs) for cardiovascular stent application. The standard published guidelines (Table 1.2) are dedicated to assess the biocompatibility of corrosion resistance materials (CRMs). Those published guidelines are based on the phenotypic measurements and do not consider the continuous release of degradation products from BMs. In this doctoral project, a new approach in assessing the biocompatibility of BMs is explored. This new approach consists of cell culture gene expression study in the presence of BMs complementing the assessment of the cellular behaviour such as cell death, adhesion, change in morphology, metabolic activity, etc. Therefore, a bottom up approach (gene expression study) is of importance to assess BMs since some genes have the potential as biocompatibility markers. Specific objectives of the project are listed as the followed:

1. To verify the feasibility of gene expression-based assessment method as an approach to evaluate BMs biocompatibility.

2. To identify potential gene markers to assess the biocompatibility of BMs.

3. To validate potential gene marker to assess the biocompatibility of BMs.

1.5 Strategy of the project

Biocompatibility markers are expected to be key parameters in predicting cellular behaviour towards metals and their degradation products. This could be useful for material scientists in conducting preliminary biocompatibility tests need on BMs. We designed an experimental setup in order to find some genes that might serve as potential markers for the biocompatibility of BMs. In this project, the experiment involved the exposure of fibroblasts to BMs degradation products at their cytostatic dose. Once the dose was obtained, it was subsequently applied to the same cells and followed by the mRNA extraction. It was then used to conduct a gene expression study using DNA microarray technique. This technique allowed us to measure simultaneously expression levels of thousands of genes and eventually identify potential gene markers. Selected markers were then used to confirm gene expression analysis results from the previous microarray. Afterwards, a gene was chosen as a biomarker to analyze the biocompatibility of BMs. Its expression was validated subsequently with real-time PCR technique and Western blot analysis for mRNA and protein expression respectively. The strategy of the project is described on Figure 1.3.

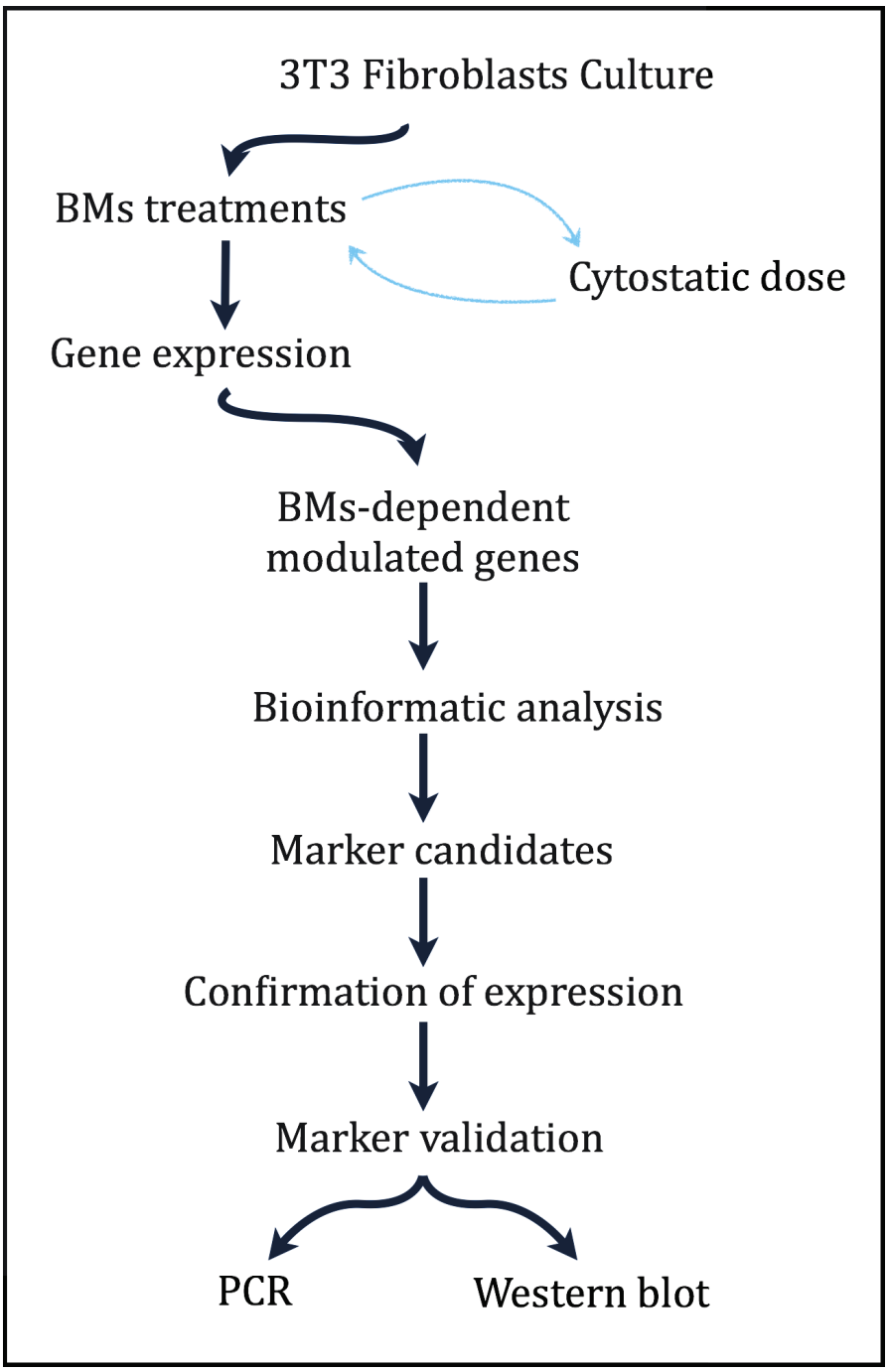


Figure 1.3. Strategy of the doctoral project. The project consists of gene expression study as a new approach to assess BMs for cardiovascular stent application. The rationale of this project was published as the first publication for the doctoral project, the second publication was the report of fibroblasts gene expression study in the presence of BMs, and the third publication was the report on marker identification and validation.

1.6 Structure of the thesis

The thesis is divided into five chapters. The first chapter consists of an introduction where the background of the study, objectives, and strategy of the project are delineated. The next chapter describes a general introduction on cardiovascular disease (CVD) and treatment that shows BMs could serve as a revolutionary treatment. The lack of pertinence for the conventional methods to assess BMs biocompatibility also described in the second chapter. It concludes the gene expression study as a new approach to assess BMs.

Chapter 3 consists of a literature review of the new approach in assessing the biocompatibility of BMs. It also describes the state of the art of both iron and magnesium as BMs. The conventional methods to assess the biocompatibility of non-corrodible metals are also covered. Moreover, the potential gene markers amongst various gene families were analyzed, leading to a conclusion of their interesting implication in confirming the biocompatibility of BMs.

Chapter 4 reports the gene expression study of 3T3 fibroblasts, which were exposed to BMs. Those degradable metals such as Fe-35Mn and pure Fe were previously developed in our laboratory. They were transformed into powder in order to provide vast surface contact with culture medium. The exposure of the BMs was then followed by the gene expression study. Bioinformatics analyses were then performed to select the potential markers.

Chapter 5 presents the investigation of caveolin gene expression in the presence of BM and metal salts. The chapter describes the consistent down-regulated expression of caveolin through the exposure of various metals from different sources. This chapter also presents how caveolin showed similar pattern of expression within different cell lines. This pattern suggests the potential use of caveolin as a biomarker to assess the biocompatibility of BMs.

Chapter 6 consists of general discussion about biocompatibility and several factors that could affect it. The mechanism of BMs biodegradation is also described and followed by the *in vitro* and *in vivo* pertinence to assess BMs biocompatibility as well as clinical study update for BMs. Conclusion for the doctoral project and perspectives are presented at the last section of this thesis followed by the references and annexes.

Chapter 2

Cardiovascular stents: Introduction, available materials and problems

2.1 Cardiovascular disease

Cardiovascular diseases (CVD) include coronary artery disease (CAD), cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis and pulmonary embolism. In 2010, CVD was responsible for 31.9% death, which equals to one of every three deaths in the United States. Thirty-four % of deaths due to CVD occurred before the age of 75 years, about 4 years earlier than the actual average life expectancy. Amongst all CVD cases, CAD killed 379,559 Americans in 2010, about 50% of diseases mortality or 1 of every 6 deaths. In Canada, about 32% of deaths were caused by CVD, in which 54% related to CAD, 25% myocardial infarction and 20% stroke. Although the death rate related to CVD has declined up to 31% from 2000 to 2010, the burden of the diseases remains high [11]. The world wide prevalent of CVD is depicted in Figure 2.1.

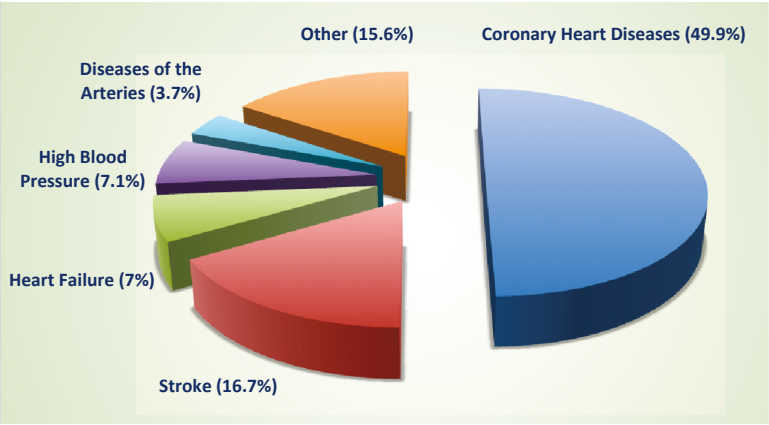
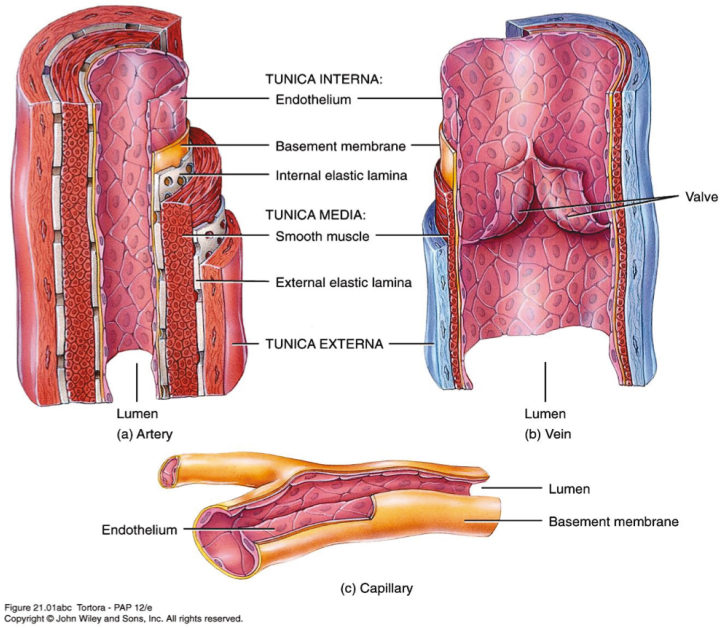


Figure 2.1. Death percentage due to cardiovascular disease (CVD) worldwide. Coronary heart diseases accounts for almost 50% death of CVDs [12].

Atherosclerosis is an inflammatory disease that characterized by the progressive accumulation of lipids and inflammatory cells within the intimal layer of the coronary artery (arterial structure is described in Figure 2.2). The progression of atherosclerosis might be initiated at an early age, as depicted in Figure 2.3. It is induced by the alteration of the endothelial layer allowing the internalisation and accumulation of LDLs (low density lipoproteins). Circulating monocytes are then recruited as they attached to the site where endothelial layer is altered. They migrate into the sub-endothelial layer where LDLs have accumulated and transform themselves into macrophages. The macrophages then scavenge ox-LDLs becoming lipid laden and subsequently converted into foam cells. Moreover, growth factors and cytokines are then released by foam cells allowing the migration of vascular smooth muscle cells (VSMCs) from the medial layer into the intimal layer. VSMCs then proliferate and produce extracellular matrix component such as collagen contributing to the formation of a fibrous cap [13].

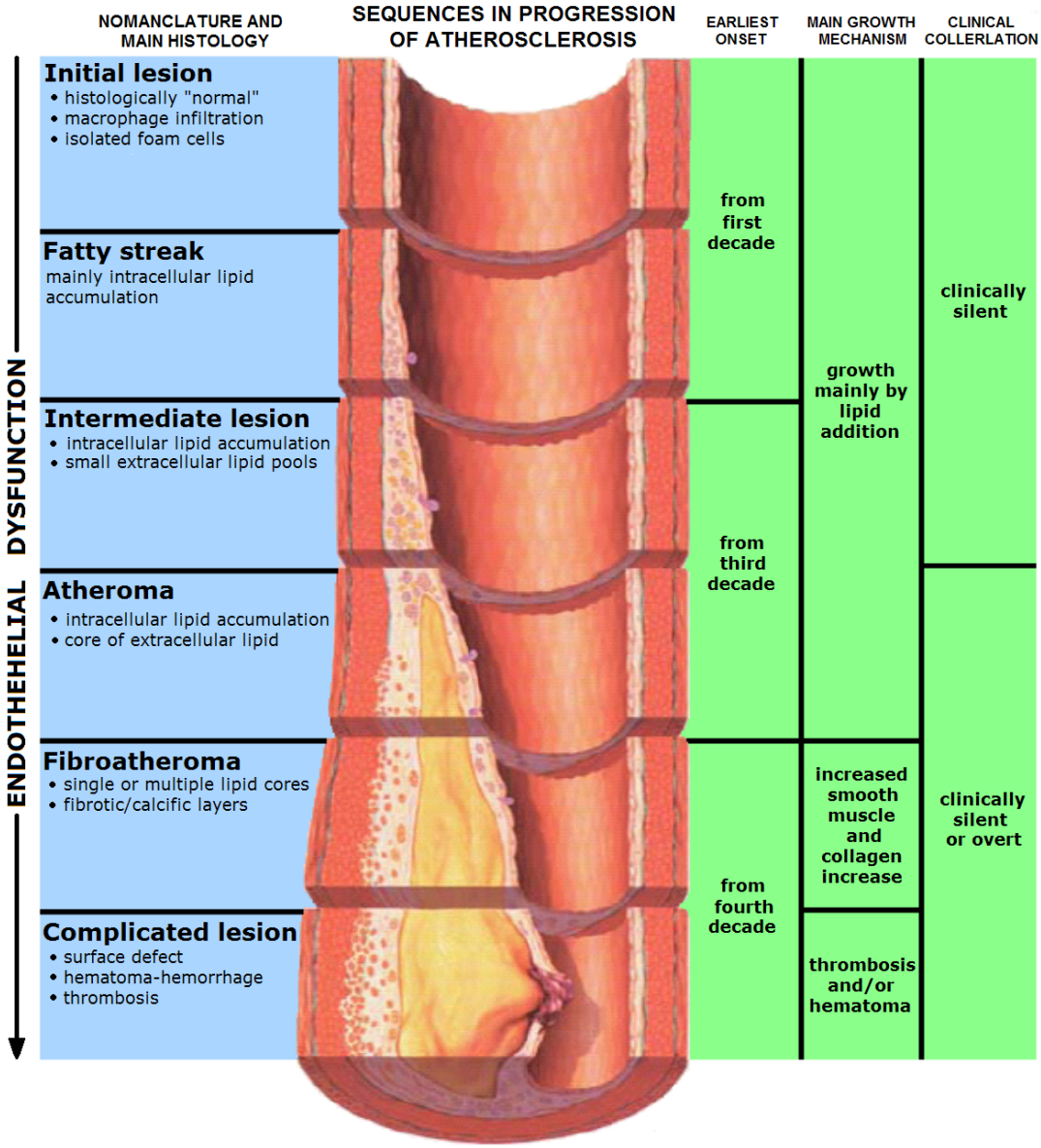


(Source: http://www.nlm.nih.gov/medlineplus/ency/18020.htm; March 2014)

Figure 2.2. Arterial and vein structure. Artery consists of tunica interna, tunica media, and tunica externa, which consist of endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblast cells (FBs) respectively. Vein has similar layer structure to that of the artery. However, tunica media of artery is thicker due to the contractibility property of the artery.

If the pathological process persists and macrophages fail to remove the accumulated LDLs, they become apoptotic and they release LDLs to the vessel wall, prothrombotic molecules, and metalloproteinase. Progression and complication of atherosclerosis are characterized by a decreased number of VSMCs as well as the formation of immature vessels making the atherosclerotic lesions more susceptible to rupture. Atherosclerotic plaque disruption and the subsequent exposure of thrombogenic substrates initiate platelet adhesion, activation, and aggregation on the altered vascular surface; and the activation of the coagulation cascade, leading to the formation of thrombus and clinical manifestations of the atherosclerotic disease, acute myocardial infarction or sudden death [13].

The prominent causes and risk factors of atherosclerosis are unknown. However, certain conditions, traits, or habits may raise the chance of developing atherosclerosis. Most of the risk factors can be controlled preventing the occurrence of atherosclerosis. Those risk factors include high total cholesterol and low-density lipoprotein cholesterol (LDL-C), low level of high-density lipoprotein (HDL) in the blood, hypertension, tobacco smoke, diabetes mellitus, obesity and sedentary lifestyle. The internal lining of artery, the endothelial layer, is more prone to physical damage in people who have more than one these factors. Once the physical damage occurs, low-density cholesterol (LDL) will enter the arterial wall and will be deposited as a plaque initiating the formation of atherosclerotic lesions as previously described [14].



([Source: http://www.nlm.nih.gov/medlineplus/atherosclerosis.html](http://www.nlm.nih.gov/medlineplus/atherosclerosis.html); March 2014)

Figure 2.3. The developent of atherosclerosis. Atherosclerosis is initiated by the endothelial dysfunction allowing the internalization of LDLs into sub-endothelial layer of the artery. Circulating monocytes are then attracted and differentiated into macrophages. They are then further converted into foam cells Moreover, growth factors and cytokines are then released by foam cells allowing the migration of vascular smooth muscle cells (VSMCs) from the medial layer into the intimal layer. VSMCs then proliferate and produce extracellular matrix component such as collagen contributing to the formation of a fibrous cap.

2.2 Treatments for cardiovascular disease (CAD)

Healthy life style is known to reduce risk factors associated with CAD. However, when

the risk of coronary artery obstruction is considered high, specific medications are then prescribed. When the occlusions exhibit potential risks that medication cannot handle anymore, clinical treatments are highly recommended in order to decrease the risks of the blockage. Percutaneous transluminal coronary artery (PTCA) catheterism and by-pass graft surgery are the clinical procedures conducted when atherosclerosis gives significant change of blood flow, as described in Figure 2.4.

Medical treatment is given to help patients with a high potential for complications from atherosclerosis or having a high risk of blood clotting. The type of the medications prescribed depends on the symptoms. For patients with developing atherosclerosis, statin is the most commonly prescribed drug, it reduces the development of atherosclerosis by reducing the LDL cholesterol thus reducing the risk of atherosclerosis. For patients with a high potential of blood clotting, antiplatelet drugs are often prescribed. Antiplatelet drugs will inhibit the formation of blood clots and prevent vessel damage and blockage. Another drug that often prescribed is β-blocker, which decreases cardiac activity by inhibiting the rate and the force of cardiac contraction [15].

Clinical treatments are conducted when atherosclerosis is leading to a potential blockage and when medication is not considered sufficient. PTCA treatment and by-pass graft surgery are the main options. They are conducted differently based on the complexity, implicated vessels, and urgency of the situation. PTCA is conducted by inserting a deflated balloon tipped wire (catheter) into the radial or femoral arteries all the way to coronary artery. Once the tip is in the narrowed artery, the balloon is inflated in order to restore normal blood circulation. If three or more arteries have to be treated or when PTCA is not an option, then by-pass graft surgery is conducted. It requires open-heart surgery (highly invasive) with higher risk of death. With by-pass graft surgery, the blood within the coronary artery is detoured to a new arterial path, which is taken from another artery (sometimes a vein) from the body [16].

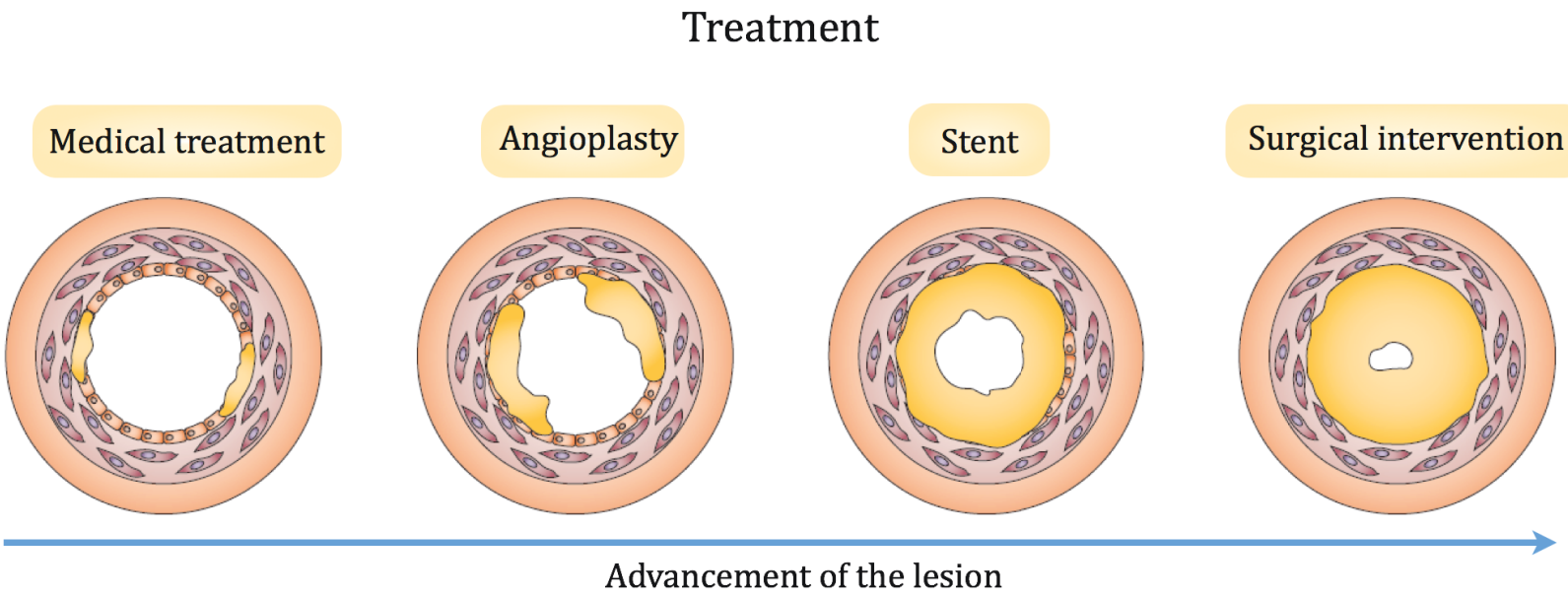


Figure 2.4. Lesion-dependent treatment for CAD. Treatment of CAD is given based on the advancement of the lesion. Medical treatment is given for the patience with a high potential for complications from atherosclerosis or having a high risk of blood clotting. Percutaneous transluminal coronary artery (PTCA) procedure is given to patients with advanced lesion. Stent deployment is now mostly applied following angioplasty intervention. Surgical intervention or by-pass graft surgery is given to the patients with complex and severe lesion. PTCA and by-pass surgery are the clinical procedures that give significant change of blood flow. Adapted from [17].

2.3 Revolutionary treatments of CAD

Practically, about 30-40% of PTCA procedures will be followed by re-occlusion (restenosis) of the treated site of artery within six months. A mesh-like tubular structure called stent has then become a standard procedure following PTCA to keep the artery opened. Cardiovascular stents range between 3-4 mm in diameter before inflation and 4-6 mm after inflation, 10-16 mm in length, and around 100 μm in thickness [18]. They are deployed by balloon inflation with the help of catheter that is inserted into the radial or femoral arteries.

Coronary stents can be made of metals or polymers. Commercially available metallic stents are made of stainless steel, cobalt-chromium alloy, and titanium, while polymer-based stents are made of poly lactid acid (PLA), poly(DTE carbonate) and poly(anhydride ester) salicylic acid [19]. Polymer-based stents are less preferable for coronary artery application since they have low radial strength which may result to early recoil after implantation, Tsuji *et al*. [20] have reported this limitation of the polymer-based stents which showed approximately a 20% recoil rate when transplanted in human coronary arteries. Furthermore, polymer-based coronary stents are often associated to a significant degree of local inflammation and they are radiolucent which require fluoroscopic visualization.

The history of cardiovascular stents is closely related to the development of angioplasty. Firstly introduced in 1964 by Dotter and Judkins, percutaneous transluminal angioplasty (PTA) was first described to treat peripheral arterial diseases. Later in 1977, Gruntzig introduced balloon coronary angioplasty—percutaneous transluminal coronary artery (PTCA), which subsequently became one of the most frequently performed non-invasive medical procedures in clinical practice today and it was remarked as the first revolutionary treatment of CVD [21], as shown in Figure 2.5. However, PTCA alone was considered not sufficient since it was often followed by acute vessel closure and restenosis. Bare metal stent (BMS) deployment was then often applied following the treatments in order to avoid the drawbacks when only balloon was applied. BMS was then considered to be the second revolutionary treatment for CAD. Nevertheless, subacute thrombotic coronary artery

occlusion was observed in up to 18% of BMS implantations. This fact discouraged the application of BMS until it was reported that anticoagulation therapy with the use of dual antiplatelet therapy and/or adequate stent deployment could assure the safety of BMS. Consequently in 1999, about 84.2% of PTCA procedures were followed by BMS stent deployment [22].

However, BMS application was not flawless. BMS deployment was subsequently reported to lead to in-stent neointimal hyperplasia, which refers to the migration of smooth muscle cells to the intima layer where they subsequently undergo over proliferation in response to foreign material—BMS. The rate of in-stent intimal hyperplasia was reported to reach 20-30% following the BMS implantations. [23] This hindrance was approached by the incorporation of anti proliferative drugs that inhibit the cytokine-and growth factor-mediated proliferation of smooth muscle cells, which reduce the rate of in-stent neointimal hyperplasia. These devices were later commonly called drug-eluting stents (DES)—the third revolutionary for CAD treatment. Although their advantage in reducing repeat revascularization has been widely reported, major concerns are still associated to their application such as late stenosis and prolonged antiplatelet therapy. Due to the contradictive application of DES, BMS deployment is still one of the alternative managements of CAD. In 1999, DES implantation was reported in more than 75% of PTCA procedure in the U.S. [24]. Nowadays, the number of DES implantation varies from one hospital to another.

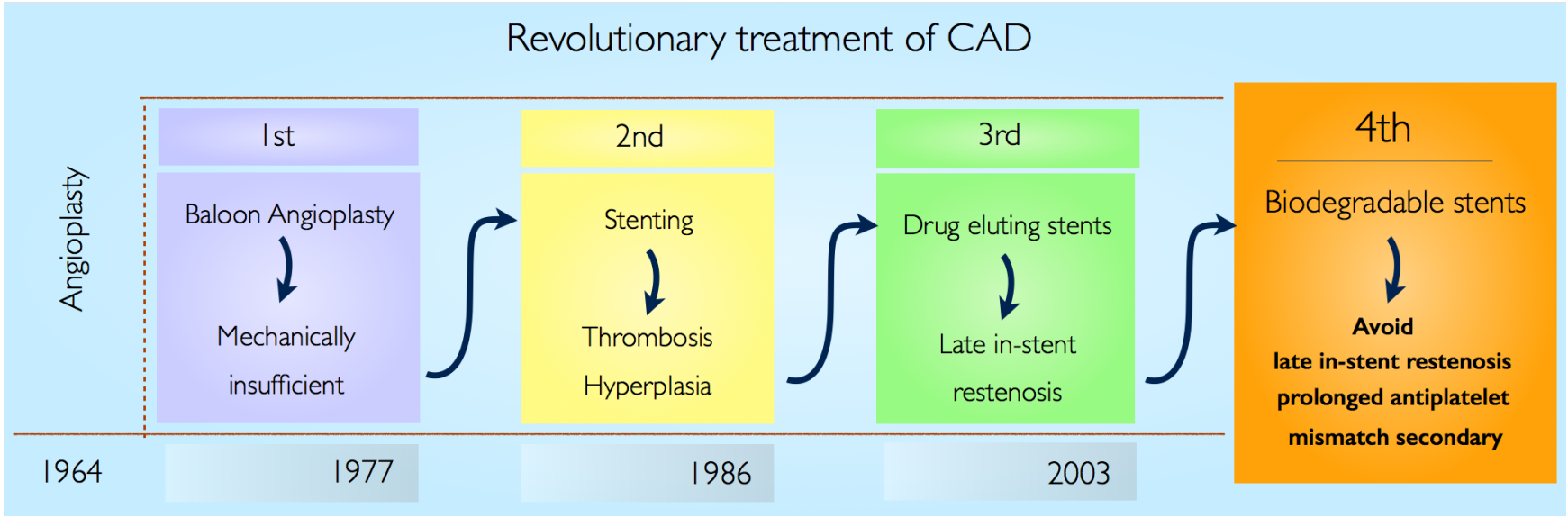


Figure 2.5. Revolutionary treatment of CAD. PTCA was considered as the first revolutionary treatment to treat CVDs and later on it was found to be mechanically insufficient. Therefore second revolutionary treatment emerged when BMS was introduced to as a standard procedure for PTCA in 1986. However, BMS deployment often followed by thrombosis and hyperplasia. Third revolutionary treatment was then discovered when anti-proliferative drugs integrated on the stent's surface to avoid hyperplasia. Nonetheless, it is often related to late in-stent restenosis occurrence once the drugs are completely eluted. For this reason, the 4th revolutionary treatment is emerging. Bioegradable metallic stent (BMS) is expected to disappear once the arterial remodelling has been completed, thus avoiding chronic disadvantages. Adapted from [25].

The first generation of DES constitutes either serolimus or paclitaxel as antiproliferative drugs with stainless steel as the bulk material. During their early development, they have been reported to significantly reduce neointinal proliferation when compared to BMS. Regardless their astonishing first impression, first generation of DES was associated with complication due to [26]:

1. Very late stent thrombosis (ST). It has been reported that very late ST occurs between 0.36% - 0.6% per year to at least 2 years after DES implantation [27].

2. Impaired endothelialisation. Antiproliferating drugs of DES might impair endothelialisation process which causes blood to stent struts and potentially induce ST. Animal study has demonstrated a significant exposed area of stent struts with the use of DES when compared to BMS (SES=3.08 mm^2; PES=3.54 mm^2; BMS=0.12 mm^2) [28].

3. Polymers-induced hypersensitivity. Polymers are incorporated on the surface of DES to deliver antiproliferating drugs. Poly(ethylene co-vinyl acetate) and poly(styrene-b-isobutylene-b-styrene) were applied on SES and PES respectively have been reported to induce localized vascular inflammation, apoptosis of SMCs, and hypersensitivity reactions both in animal models and human [29].

Second generation of DES (DES II) came up to ameliorate the former generation. DES II is made of cobalt chromium (Co-Cr) alloy which presents superior radial strength and radio opacity compared to stainless steel. This allows for thinner struts and improved stent deliverability. DES II eludes limus drugs and uses more biocompatible polymers such as phosporylcholine—a natural component of cell membrane. The rate of endothelialisation for DES II was reported to reach 99.9% after 3-month implantation, comparable to stainless steel-based BMS [30]. However, its performance in reducing very late ST is more appropriate when compared to SES and PES. Prolonged anti platelet therapy still remains unavoidable for DES application [31].

2.4 Biodegradable stents, 4th revolution in CAD treatment

The use of new implant materials may address the drawbacks of DES. Corrosion could constitute an advantage for stent material while it was considered to be a disadvantage in permanent materials. Corrosion nature of a material will lead to the elimination of the implant, which could relate to with the temporary need of coronary artery stent. When a stented artery remodelling is completed, there are little reasons for the stent to permanently remain. This new paradigm has led the idea of "biodegradable coronary artery stent" in the field [6]. In this document, biodegradable metals (BMs) refer to materials with the ability to degrade within a living system, while their counterpart is corrosion resistant. The BMs in this text refer to the pre-existing materials for biodegradable metallic stent (BMS).

The use of biodegradable coronary stents could help some problems associated with the current generation of DES. Late thrombosis could be reduced since the stent would completely disappear from the artery. Secondly, the self–occlusion of the stented artery could be avoided when the stent is implanted in a growing coronary artery. And finally, toxicity of the corrosion products of the stent materials is unlikely since the available biodegradable stents could be fabricated from metal elements such as magnesium and iron with relatively low toxicity. These new features that BMs have to offer apparently could lead the intervention to the 4th revolutionary treatment for CAD management.

2.4.1 Magnesium as a BM for cardiovascular stent application

High allowable daily intake for Mg (about 400 mg/day; US FDA) makes the systemic cytotoxicity of Mg unlikely. However, pure Mg is considered to be chemically reactive and to have poor mechanical properties for stent application. Hence, it is often alloyed with elements such as calcium (Ca), Zinc (Zn), strontium (Sr), zirconium (Zr), rare earth elements, and aluminum (Al) to decelerate its corrosion rate and to improve its mechanical properties [32]. Despite the wide variability of elements that have been

alloyed with Mg, few are considered to have potentials pursuing all the way to the clinical stage. Among the most recent applications of Mg for stents is the use of Mg-6Zn alloy for common bile duct biodegradable stents and tested in rabbit model up to 3 weeks implantation period [33]. The *in vivo* experiments showed that Mg-6Zn stents did not affect several important biochemical parameters or harm the function or morphology of the bile duct, kidney, pancreas and liver, and therefore suggested that the alloy is a safe biocompatible for common bile duct. There were at least two latest reports on clinical studies of Mg stents. First, the Absorbable Metal Stent Implantation for Treatment of Below-the-Knee CLI (AMSINSIGHT) clinical study involving 117 patients with 149 CLI lesions that revealed a comparable results between those treated with PTCA and PTCA+ stenting and concluded that the stent's efficacy in long-term patency over standard PTCA in the infra-popliteal vessels was not evident [34]. Second, the Clinical Performance and angiographic Results of Coronary Stenting with Absorbable Metal Stents (PROGRESS-AMS) where 71 Mg stents were implanted in 63 patients and revealed that the stents can achieve an immediate angiographic response similar to that of other metallic stents and be safely degraded after 4 months [35]. However, all *in vivo* and clinical studies on Mg stents suggested the necessity to prolong degradation time.

2.4.2 Iron as a BM for cardiovascular stent application

High blood Fe content (400–500 mg/L) and high allowable daily intake (up to 40 mg), which about the weight of the whole stent, make the toxicity of iron stent arguable [18]. Among the most recent application of iron for stents is the assessment of the safety and efficacy of Fe stents was conducted by a short-term implantation of Fe and Co–Cr (control) stents in the coronary arteries of juvenile domestic pigs [36]. The results showed that the intimal thickness, intimal area, and percentage of occlusion were better for the Fe stents which lead to a conclusion that Fe stents were relatively safe. Long-term implantation of Fe stents in the descending aorta of mini-pigs that showed no difference in the amount of neo-intimal proliferation between SS316L

(control) and Fe stents, no sign of Fe overload or Fe-related organ toxicity as well as any evidence for local toxicity due to corrosion products [21]. Although iron has a comparable strength and ductility to those of stainless steel, its degradation rate is considered too slow for stent applications. Slower degradation rate of Fe-based alloys within *in vivo* system is mainly caused by the appearance of passive layers (oxides or phosphates) that blocks further oxidation process [8].

2.4.3 Zinc as a BM for cardiovascular stent application

Zinc was firstly use as an alloying element for Mg. Its presence within the alloy was tolerable as such 50 wt% Zn in the alloy was still appropriate as a biodegradable implant [37]. However, the use of Zn within the context of biodegradable implants is relatively new. Zn-based alloys could be preferable over Mg-based alloys since they can be fabricated by classical routes such as die casting and hot rolling. Moreover, they have lower melting point, lower reactivity, and superior machinability compared to those of Mg-based alloys. Zinc alloys with up to 3 wt% Mg was recently investigated for bone fixation applications [38]. More works have to be done on Zn-based alloys to confirm their suitability as biodegradable metals.

2.5 Material biocompatibility for stents

Biocompatibility assessment is needed in order to make sure that the materials for stent fabrication will not promote an inflammatory response or systemic toxicity. The assessments include non-functional tests, in which the materials have not fabricated into the stent yet; and functional tests, in which the materials are in the form of stents and tested in the artery. In fact, non-functional and functional tests are compulsory since the beginning of the material development. This delicate work is required in order to provide proofs of safety to the regulatory agency such as FDA (Food and Drugs Administration of USA) and to the patients prior to release to the market.

Biocompatibility is a compulsory requirement for todays implants. It refers to the ability of a material to perform a particular function within living tissues and is followed by appropriate host responses—minimum inflammatory and toxicity reactions both locally and systematically. Nowadays, stainless steel is known as a gold standard for implant materials. Therefore, available standards to assess the biocompatibility are all attributed to corrosion resistant materials. As BMs are now considered as a potential future for cardiovascular stent application, assessment standards need to adopt BMs.

Guidelines for biocompatibility assessment of new candidate implant materials are provided by the ISO (International Standard Organization) as guided by ISO-10993 part 1-20 and ASTM 1983 [39]. The tests consist of *in vitro* and *in vivo* assays with respect to cytotoxicity, hemocompatibility, mutagenicity, hypersensistivity, genotoxicity, carcinogenicity, sensitivity and systemic effects. As described in Figure 2.6, those assays are devoted for corrosion resistant materials and are not specifically addressed to assess BMs. Moreover, the year that the guidelines were made—1983, is considered to be no longer suitable with materials development nowadays. However, they have potentials to be used to assess BMs.

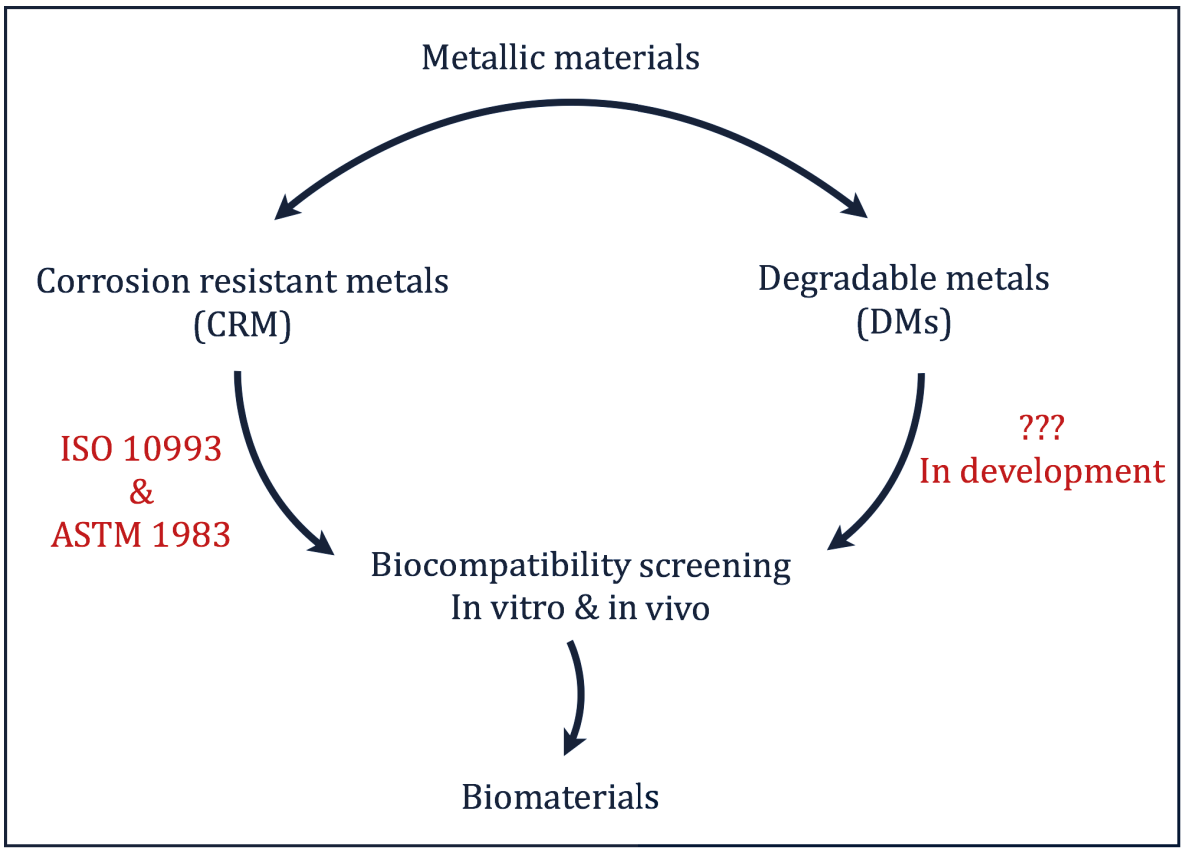


Figure 2.6. Flowchart of metallic materials assessment prior to acceptation as biomaterials. Biocompatibility assessment for corrosion resistant materials is guided by ISO 10993 and ASTM 1983 standards. However, assessment standard for BMs is in development as the mentioned standards are not adopted to test BMs. Adapted from [6].

2.6 Common testing methods for safety assessment of stent materials

The assessments for implantable materials safety are guided by the ISO-10993 standard. These guidelines have been used to assess the available corrosion resistant materials. The following tests are the most regularly conducted for metals and polymers. Giving the spotlight to stainless steel 316L, it has been tested numerous times in order to confirm its biocompatibility yet also has been employed as the reference material in developing new biomaterials such as titanium and iron based alloys. The summary of conventional tests is described in Figure 2.7.

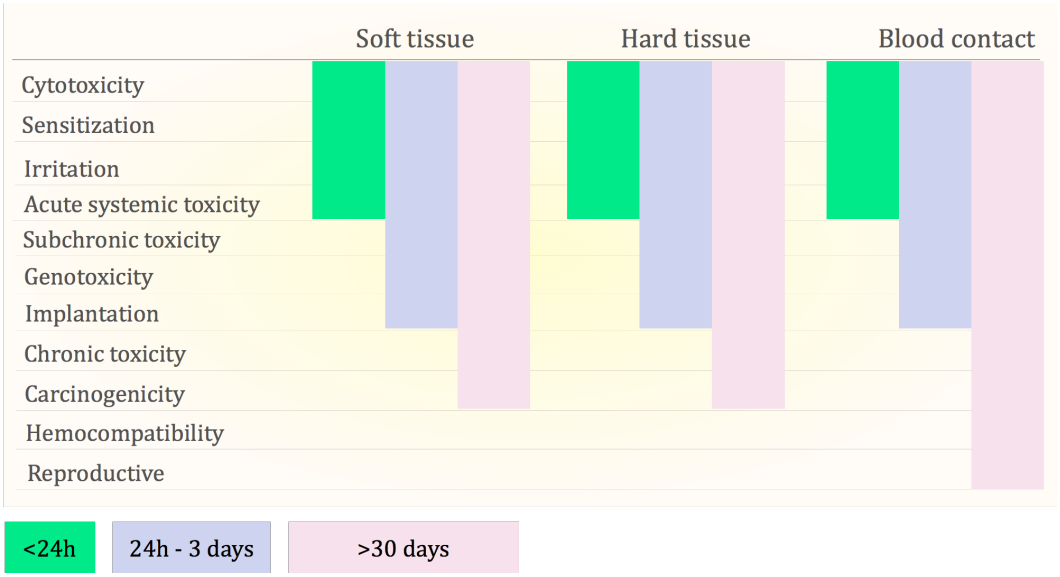


Figure 2.7. Common testing requirements for corrosion resistant materials (CRMs) based on ISO 10993 and ASTM 1983 standards. Depending on the implant site, CRMs are required to be assessed based on the listed test. Blood contact CRMs need to meet the requirements for hemacompatibility and reproductive-related assessments. Adapted form [25].

2.6.1 *In vitro* tests

Nearly six decades ago, tissue culture was limited as an exclusive work of art. It was then changed as an investigative tool in biological science pioneered by C.M. Pomerat. The development of tissue culture technique was reinforced by the invention and the advancement of synthetic culture medium and antibiotics. Afterwards, it has made its way as a screening tool for toxicity testing of various drugs, chemicals, and materials [40]. Tissue culture has been used as a preliminary testing method because of its relatively low-cost and that is less time-consuming comparatively to *in vivo* experiments. It can involve culture of cells in direct contact with the tested material or the use of soluble extracts of the material. *In vitro* testing methods provides an opportunity to 'tailor' new ways to assess biomaterials. However, it is important to notice that *in vitro* experiment cannot replace the subsequent *in vivo* stages. Both *in vitro* and *in vivo* tests are needed to predict the biocompatibility of biomaterials. Conventional methods to assess BMs are described bellow [2]:

2.6.1.1 Cytotoxicity tests

A. Elution test

Elution test is considered to be a suitable method to determine the toxicity of leachable biomaterials. The materials are extracted or eluted with cells culture medium. Serial dilution of the extract is then incubated with cultured cells for 24-78 hours periods to determine toxic doses.

Cell proliferation is then determined through direct counting, radiolabeled thymidine, tetrazolium salt conversion or deoxyribonucleic acid (DNA) quantification. Membrane permeability can be also measured to evaluate cell damage, this measurement applies the uptake of viability dies (neutral red and trypan blue) or the release of intracellular enzyme such as lactate dehydrogenase. In general, a 50% decrease of the measured parameter compared to control group is considered to be severely toxic.

B. Agar overlay

Agar overlay method is used to evaluate the toxicity of the diffusible components of a material. The method is said to be good for materials having a high density that could crush or damage cells if put in direct contact. This method involves the placement of the materials on agarose layer which is covering the cells for 24 hours. The toxicity of the diffusible components to the cells is determined by the addition of viability staining solution followed by the measurement of the dead zone of the cells surrounding the tested materials.

C. Direct contact test

Here, cells are cultured directly on top of the materials or placed under the materials. Cell proliferation or growth can be then measured as described above. The measurements applied to this test are the same with elution test. Cell morphological changes, intracellular molecules quantification (ATP and signaling molecules), and the release of chemokine/cytokine and total protein production can also be evaluated.

2.6.1.2 Hemocompatibility tests

A. Hemolysis assay

Hemolysis assay is generally conducted with materials put directly contact with blood. This test measures the hemoglobin released spectrophotometrically after materials are incubated with red blood cells isolated from rabbits, mice, or rats. The red blood cells concentration and the volume-to-surface ratio of the materials and red blood cells must be carefully controlled in order to have good reproducible results.

B. Clotting assay

In the clotting test, the materials are exposed to whole blood serum. The result is reported by comparing the time of clots development for tested materials with respect to the reference material. The main issue of this test is that the design of the materials being tested will effect the clotting time due to the change of blood flow (turbulent flow may increase the hemolysis and clotting).

2.6.1.3 Mutagenicity test

Ames test is the preliminary screening test used to identify potential mutagens. This test employs mutant bacterial culture (*Salmonela typhimurium*) which requires histidine to grow. If the materials somehow change the genotype of the bacterial culture, the bacterial culture will survive to grow in histidine-free environment.

2.6.1.4 Hypersensitivity test

Hypersensitivity IV (T-cell mediated sensitivity) is often used to observe the immune response of foreign materials. Hypersensitivity test is considered to be more to individual response test rather than general immune response induction. This test was successfully developed by Merrit et al. [41] which have been able to show the presence of antibodies to albumin-metal complexes (haptens) in patients with well functioning cobalt-based alloy joint replacement components.

2.6.2 *In vivo* tests

In vivo tests comprise both non-functional and functional tests. Non-functional tests employ material samples exposed to animals' soft tissue (intramuscular, subcutaneous and intraperitoneal), histological observation is then conducted and compared to the reference materials. Functional test is then conducted to evaluate how the materials will perform at the implant site where the materials will be employed.

2.6.2.1 Genotoxicity

Gene mutations (base-pair mutations, frame-shift mutation and deletions), chromosomal aberration and DNA effects can be tested for genotoxicity evaluations. Genotoxicity tests are needed to show whether degradation/leachable products of the materials induce genomic alteration, this is done especially when *in vitro* tests show potential genotoxicity of the materials or their components.

2.6.2.2 Carcinogenicity

Carcinogenicity tests are considered when biomaterials will be used for permanent applications. This test requires a long period of time implantation in regard to the animals' life span, thus, rodents are regularly used as models.

2.6.2.3 Sensitization

Redness (erythema) and swelling (edema) are the two parameters used to score the hypersensitivity reaction of biomaterials in animal tests. Guinea pig is the most employed animal for this test since its reaction the most resembles the immune response of the human body. The materials or the extract of materials are placed or injected subcutaneously and followed by 1-2 weeks observation scoring erythema and edema.

2.6.2.4 Irritation

The irritation test is similar to the sensitization test, but the immunological response is not involved here. The test applies the injection of the extract materials intracutaneously followed by 24, 48 and 72 hours observations of erythema and edema.

2.6.2.5 Systemic Effect

With systemic test the biomaterials are evaluated for their capability to cause organs or tissues damage far from the implantation site. The test is important if degradation products from the biomaterials enter the circulation system. Adverse effects can be defined as: acute (<24 hours), subacute (14-28 days), subchronic (10% of an animal's life span) and chronic (longer than 10% of an animal's life span).

2.7 Cell line choice for biocompatibility assessment

2.7.1 Fibroblasts

Arteries consist of three different cell types, namely the fibroblasts, vascular smooth muscle cells, and the endothelial cells. The fibroblasts layer is the most outer structure of the artery. In the early beginning of cell biology development, fibroblasts were considered as a monotonous population of cells and there was not given much attention. This view has fundamentally changed, fibroblasts are now considered as a central component of tissue biology. Fibroblasts are the main cellular constituents of the connective tissues such as tendon, ligament, and skin; and are present virtually in every organ. They are responsible for the production of collagens, the principal component of the extracellular matrix (ECM). They are also responsible for the production of various growth factors (TGF-β) and cytokines (TNF-α), as well as matrix degrading-enzyme, MMPs (matrix metalloprotease) [42]. This allows fibroblasts to play a major role in tissue remodelling and wound healing processes. Fibroblasts also help organizing ECM by producing proteoglycans. Proteoglycans are able to interact with other matrix molecules, having complex molecules with a protein core covalently attached to complex carbohydrate glycosaminoglycan chains.

Fibroblasts are mechanoresponsive cells, able to convert mechanical signals into biological events such as expression of numerous genes. The mechanoresponsive genes are largely categorized into two types, ECM-related genes including collagens (Col-I, Col-III, and Col-XII), matrix metalloproteases (MMP-1, MMP-3, MMP-13), connectin, cystatin, calmodulin, and TGF-β; and inflammation-related genes such as COX-2 and mPGES-1. Fibroblasts have been widely employed for biocompatibility assessment procedures of various metallic elements such as arsenic and silver, and also various alloys such as gold-palladium and cobalt-chromium-molybdenum alloys [43]. The surface area of metal in contact with cell culture medium influences the viability of fibroblasts. It was also found that arsenite could damage DNA repair signalling pathway by altering a tumor suppressor gene (p53) function. P53 can be induced by various cellular stress conditions such as ionizing radiation or DNA damage. It acts as anti-proliferative and can either promote cell arrest to allow DNA

repair, or apoptotic cell death when the damage is overwhelming. Experiment that involved metal exposures on fibroblasts has revealed a gene that represents a target for cadmium and platinum-mediated gene activation, and also a metal-responsive gene that could be a target for broader range of metals, favouring metallic assessment method within genetic level through fibroblast gene expression pattern [44].

2.7.2 Smooth muscle cells (SMCs)

Smooth muscle cells are most prevalent in the medial layer of the artery. They have both contractile and secretory properties. Their contraction is responsible for the modulation of vessel diameter and therefore blood distribution and pressure. They can secrete numerous growth factor and chemokines such as IGF-1, β-FGF, TGF-β, TNF-α, IL-1, PDGF, etc. depending on the presence of stimuli such as inflammation. Additionally, SMCs may have phenotypic modulation since they are not terminally differentiated in mature organism. Differentiated SMCs (quiescent state) proliferate slowly and express specific and unique sets of contractile proteins, ion channels, and signalling molecules. However, in response to inflammatory mediators, growth factor inhibitors, mechanical influences, cell-cell and cell-matrix interaction, they are able to increase proliferation rate, migration, and synthetic capacity known as proliferative state. The later state has been shown to have low expression of specific contractile-markers, but higher production of ECM, especially collagen III and fibronectin as well as MMP-1 and MMP-3. This versatile property plays an important role in vascular remodelling, imbalanced function inevitably leads to wall dysfunction and vascular disease. In general, quiescent SMCs are smaller than the proliferative ones, they have spindle-shaped elongated morphology and express contractile-specific proteins inexhaustibly. Common markers that are used to identify differentiated SMC include SMC-a-actin, SM-MHC, calponin, caldesmon, metavinculin, telokin, and smoothelin [45].

Smooth muscle cells have been used to assess metallic elements such as cadmium, iron, and tungsten as well as alloys such as stainless steel and magnesium-based alloys [46]. It was found that cadmium could promote the proliferation of SMCs through calcium-dependent pathway, while iron and Mg-Li-Al-RE inhibit the proliferation of SMCs. For cardiovascular stent application, inhibiting the SMCs proliferation is preferable since stent deployment is related to a 25% rate of in stent restenosis, which is observed within bare metal stent application [47]. In stent restenosis involves the migration of SMCs into intimal layer towards the stented site and followed by excessive proliferation of SMCs with this layer due to the presence of foreign material—stent. Subsequently, the diameter of luminal artery will be decreased, going against the original purpose of stent deployment.

2.7.3 Endothelial cells (ECs)

The endothelial cell layer or endothelium shields the luminal surface (intimal layer) of the artery, separating smooth muscle layer from circulating blood. Endothelial cells have a distinctive structure due to their diverse functions and properties such as vascular tone, selective permeability, metabolism, and angiogenesis. The apical surface is selectively permeable to some ions, macromolecules, and water. Endothelial cells form continuous monolayer linked by tight junctions. Generally, similar characteristics are found in endothelial cells from different parts of the body, however, this is not the case for all endothelial cells. Renal glomerular cells are found to be very permeable while the endothelial cells of brain tissue shows none and little permeability to liquid. Endothelial layer in hemapoietic tissues, spleen, and liver is found to be discontinuous with large gap (100-200 nm) permitting the transport of large sized molecules. In contrast, endothelial layer in renal glomeruli and endocrine glands is continuous which contains a thin membrane (diaphragm) and 50-60 nm transcellular pores, serves as a molecular filter. Gene markers have been used to differentiate endothelial cells, large vessel endothelial cells express copious ECM molecules such as fibronectin, osteonectin, collagen 5a1, and collagen 5a2, whereas

microvascular endothelial cells have a high expression of laminin, collagen 4a1, and collagen 4a1 [48].

The endothelial layer plays an indispensable role in hemostasis as arterial inner vasculature shield. Endothelial maintains hemostasis by producing nitric oxide (NO) and prostacyclin, which prevent platelet activation. Heparin sulphate is also found on the endothelial surface acting as the activator for antithrombine, which blocks the coagulation cascade by preventing the transformation of prothrombine to thrombine. Once an injury occurred and endothelial layer is broken, NO, prostacyclin, and heparin sulphate are absence; and with the presence of exposed collagens, platelet activation will occur and followed by the activation of coagulation cascade. Interestingly, endothelial layer also exhibits procoagulation property. Within the extracellular matrix of interendothelial space, tissue factor (TF) can be found, once it is released due to the injury, it activates factor X. Activated factor X will catalyze prothrombin to thrombin, and fibrinogen to fibrin subsequently [49].

Endothelial cells have been used to assess biocompatibility of various metals such as stainless steel and titanium alloys. They are considered as one of the important cell types, especially in testing cardiovascular stent materials. Endothelial cells are the first cells that undergo physical contact with stent material. They are then expected to reconstruct the broken layer and cover the material at the same time, allowing natural vascular inner lining and prevent thrombosis. Endothelial cells also contribute to inflammatory process through the expression of adhesion molecules such as intercellular adhesion molecule (ICAM-1) and vascular adhesion molecule (VCAM-1). ICAM-1 and dVCAM-1 expression was related to the surface property of Ti-6Al-4V alloy. The expression of intercellular adhesion molecule (ICAM-1) was significantly higher to all Ti alloys surface (control, furnace- and plasma-treated), while no significant expression was observed for vascular cell adhesion molecule (VCAM-1) compared to that of the control cells. Moreover, endothelial cells were also reported to have an enhanced proliferation rate when cultured on the NiTi alloy, which has been heat-treated at 600^{o}C. Nickel ions were displaced to sub-surface area due to the thick formation of TiO_2 after the heat-treatment [50].

2.8 Pertinence of conventional methods towards BMs

Biocompatibility tests of BMs have to take into account the release of metallic ions as degradation products. The release of metal ions creates imbalance charges or oxidative stress and could form complexes with metal chaelators prior to the formation of reactive oxygen species (ROS). The presence of ROS is closely related to DNA damage which can lead to cell death or cellular over proliferation. These toxicity events can be attributed to the alteration of genetic regulation inside the cells due to the released metal ions. Therefore, genetic regulation plays important roles in dealing with the degradation products [6].

Detrimental effect of degradation products could be strong enough to damage cells and may be easily observed morphologically. This is what the conventional methods are most likely able to do as shown in Figure 2.8. In a particular case, detrimental effect of the degradation products could be invisible morphologically even though the cells are internally disturbed. The disturbance may stay inside the cells during a certain time and eventually will lead to severe chronic damage. For this, the conventional methods to assess the biocompatibility of BMs need to be adjusted in a way that continuous release of degradation products is taken into account. Assessing the biocompatibility of BMs can be performed through the basic mechanisms of cellular response towards degradation products as foreign materials. Altered cellular behaviour driven by the genetic regulation can be used as a new approach to investigate the biocompatibility of BMs. Therefore, it enriches the conventional methods with more sensitive detection limit towards the cellular state in the presence of the degradation products. Hence, chronic damages can be avoided [6].

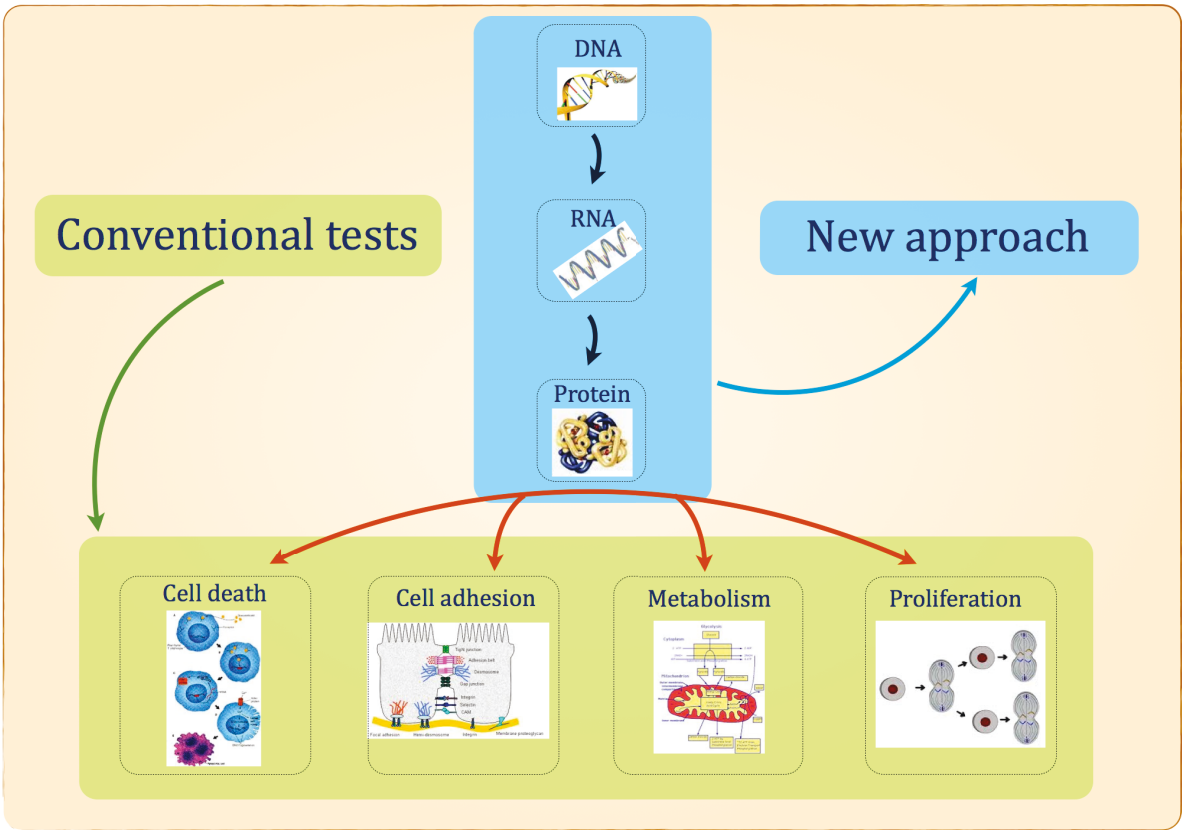


Figure 2.8. Conventional versus the new approach assessment tests. Conventional tests measure only phenotype appearance of the cells such as cell death, cell adhesion, metabolism, proliferation, etc. Meanwhile, the new approach deals with deoxyribonucleic acid (DNA), genetic substance that controls phenotype appearance [6].

Chapter 3

Assessing the biocompatibility of degradable metallic materials: State of the art and focus on the genetic regulation potential

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3.1 Abstract

For decades metallic biomaterials have been designed, developed and implanted to be corrosion resistant once implanted into the human body. Recently, biodegradable metals (BMs) have been introduced and proposed for some specific applications, including paediatric, orthopaedic, and cardiovascular applications. BMs are expected to disappear through corrosion process after fulfilling structural support for a certain period of time depending on the application site. In the past decades, a wide and rather complete set of *in vitro*, *in vivo* and for some cases also ex-vivo tests have been proposed and exhaustively investigated for the conventional corrosion resistant metallic biomaterials. Therefore, world standard and regulation bodies in the United States, Japan and Europe have developed standard and regulatory tests to accredit corrosion-resistant metals to the role of "biomaterials". This is not the case for BMs. Once implanted, these new class of biomaterials is expected to support healing process of a diseased tissue or organ while degrading with a potentially adjustable degradation rate. In this context, the same tests that the ones developed for corrosion-resistant metals cannot be simply transposed. They can be adapted in some cases while in some others their expected unique properties should inspire and lead to the design and the development of new specific tests. The current challenge is how to assess the tolerance of surrounding tissues and organs to the presence of degradation products. This work precisely focuses on this topic. The more recurrent tests leading to the assessment of the biocompatibility of conventional corrosion-resistant metals are briefly reviewed. Then, genetic regulation is proposed as an original and new approach to assess the biocompatibility of BMs. This method might provide prediction of cell behaviour in the presence of degradation products that closely related to DNA damages. Various genes have been related to the toxicity and inflammatory responses indicating their role as biomarkers to assess degradation products toxicity. Finally, some gene families that are potential to be applied as biomarkers against degradation product toxicity are summarized.

Keywords: Degradable metallic materials, degradation product, degradation test, biocompatibility, genetic regulations.

3.2 Résumé

Pendant des décennies, les biomatériaux métalliques ont été conçu, développé et implanté pour résister à la corrosion une fois implanté dans le corps humain. Récemment, biomatériaux métalliques dégradables (MBs) ont été mis en place et proposé pour certaines applications spécifiques, notamment en pédiatrie, orthopédie, et les applications cardiovasculaires. MBs ont été attendu à disparaitre à travers le processus de corrosion après avoir rempli un support structurel pour une certaine période de temps. Dans les dernières décennies, les tests de *in vitro*, *in vivo* et dans certains cas aussi *ex vivo* ont été proposées pour les confirmer la biocompatibilité des matériaux métalliques résistant à la corrosion. Par conséquent, les organismes de standardisation aux États-Unis, au Japon et en Europe ont élaboré pour déterminer des tests standard qui accréditent les matériaux résistant à la corrosion comme «biomatériaux». Ce n'est pas le cas pour les MBs. Une fois implanté, cette nouvelle classe de biomatériaux est prévue de soutenir le processus de guérison d'un tissu ou d'un organe malade lorsque ca dégrade avec un taux de dégradation potentiellement ajustable. Dans ce contexte, les mêmes tests que ceux développés pour les métaux résistants à la corrosion ne peuvent pas être simplement transposées. Ils peuvent être adaptés dans certains cas, mais dans certains autres, les propriétés uniques de MBs devraient inspirer et conduire la conception et la mise au point de nouveaux tests spécifiques. Le défi actuel est de savoir comment évaluer la tolérance des tissus environnants et les organes à la présence de produits de dégradation. Ce travail se concentre précisément sur ce sujet. Les tests les plus récurrents menant à l'évaluation de la biocompatibilité des classiques résistant à la corrosion des métaux sont brièvement passées en revue. Ensuite, la régulation génétique est proposée comme une approche originale et nouvelle pour évaluer la biocompatibilité des MBs. Cette méthode peut donner de prédiction du comportement des cellules en présence de produits de dégradation qui sont étroitement liées à des dommages l'ADN. Différents gènes ont été associés à la toxicité et les réactions inflammatoires indiquant leur rôle en tant que bio-marqueurs pour évaluer les produits de dégradation toxiques. Enfin, certaines familles de gènes qui sont susceptibles de s'appliquer en tant que bio-marqueurs contre la toxicité des produits de dégradation sont résumées.

3.3 Introduction

The scientific knowledge about tissue-implant interactions, tissue engineering, regenerative medicine as well as recent advances in cell biology, physiology, and molecular biology raises a new concept of bioactive biomaterials, rather than conventional concept of inert biomaterials, supposed to promote positive interactions with the physiological implantation sites. Degradable biomaterials constitute a novel class of considerably bioactive biomaterials which are expected to support healing process of a diseased tissue or organ while slowly degrade thereafter. The study of innovative degradable biomaterials is one of the most interesting research topics at the forefront of biomaterials in present days. The advancement of materials science and engineering, especially for processing and thermomechanical treatments, allow the structural material adjustment to meet the expected properties of materials. The increasing society expectation (demand) and the growing ageing population for a better quality of life have pushed biomaterialists to develop new technologies to provide better implants with higher clinical performance. The paradigm stating that implants must be inert and corrosion resistant has now been broken by the advent of this new class of metallic biomaterials. To clarify the identity of these two class of metals, the term “permanent” opposed to “degradable” biomaterial will be used to address corrosion-resistant metallic biomaterials.

However, the concept of degradable biomaterials is not new, degradable biomaterials have been proposed since 1988 [51]. Among the polymers that have been proposed were poly-l-lactic-acid (PLLA) [52], polyglycolic acid/polylactic acid and polycaprolactone [53]. Meanwhile, the idea of considering metals as degradable biomaterials is much more recent, even though the use of BMs for biomedical applications dates back to 1938 when magnesium was used for fixing bone fracture [54]. Metals are more mechanically interesting compared to polymers for load bearing implants such as internal bone fixation screw and plate and coronary stents. Up to now, two classes of metals have been proposed: magnesium- and iron-based alloys. Several Mg-based alloys have been investigated, including AE21 [55], AM60B [7], AZ91 [56], and WE43 [57]. Meanwhile, the potentiality of Fe-based alloys, mainly for

cardiovascular application has been also studied, including pure Fe [18] and Fe-manganese (Fe-Mn) alloys [9].

The presence of biomaterial within the body, either inert or degradable, induces reactions from the surrounding tissues called host responses. These host responses have been used as parameters to assess the biocompatibility of a biomaterial. A biocompatible biomaterial is expected to show minimum inflammatory and toxicity reactions both locally and systematically [2]. The established guidelines to assess the biocompatibility of a new candidate of permanent implant materials are provided by the International Standard Organization (ISO), American Society of Testing and Material (ASTM), etc. which consist of *in vitro* and *in vivo* tests prior to clinical human study [58]. These guidelines can be used to evaluate the cytotoxicity of degradation products which could be generated from the materials. The tests emphasize the presence of degradation products which should be negligible and in the limit of tolerance for corrosion resistance and degradable materials respectively.

As BMs are expected to disappear through degradation process, degradation products are already expected to be present in surrounding tissue as well as organs and systemic tissues, especially metabolic ones. The presence of degradation products resulting from the corrosion process affecting the metal once in contact with biological environment have the potential to generate reaction for which mechanisms are new, even unexpected, and probably unknown. Thus, conducting biocompatibility tests is probably not as much pertinent as corrosion-resistant biomaterial: investigating the toxic potential and inflammatory response of surrounding tissues based on the probability of released elements or simply physical contact between the cells and metals. The challenge is to assess the tolerance of surrounding tissues in the presence of degradation products. In other words, the biocompatibility assessment of BMs is evaluated with the same approach used for “permanent” metals though advance assessment is needed to predict the cells behaviour in the presence of degradation products.

This work aims to review the usual tests leading to the assessment of the biocompatibility of conventional corrosion-resistant metals and to propose gene expression profiling as an original and new approach to assess the BMs biocompatibility since it is often the first mechanism of cellular response. By this mean, the biocompatibility of BM is expected to be more predictable prior to classical assessment methods. Important biocompatibility features such as cytotoxicity, carcinogenicity, inflammatory response, etc. could eventually be checked by evaluating the responsible genes for each feature. In order to establish this goal, gene expression profiling results need to be calibrated and confirmed with those from the classical ones as detailed in section 3 of this article.

3.4. Assessing biocompatibility of BMs

The main issue of BMs is the degradation products that might induce local or systemic toxicity. However, toxicity issues seem to be unlikely since two common BMs—iron and magnesium, are relatively non-toxic and has been used in the development of cardiovascular stent application. Magnesium plays important roles in genomic stability, bone structure, energy generation, phospolipid and protein structures [32]. Iron plays a significant role in transport, storage, and activation of molecular oxygen, reduction of ribonucleotides and dinitrogen, activation and decomposition of peroxides and electron transport [59]. Therefore, state of the arts of both magnesium and iron are considered to be interesting BM samples to be presented.

3.4.1 Magnesium: State of the Art

Mg and its alloys have been broadly used in transport and aerospace industry mainly due high specific strength to weight ratio (ρ = 1.74 g/cm^3). The main drawbacks of Mg and its alloys for these applications are low corrosion resistance, low elastic modulus (41-45 GPa) and limited strength and creep resistance at elevated temperatures [59]. The use of Mg has been reported to be 360.000 tons per year in 1998 with the growth rate of 7% per year [60].

Mg is an essential element to human body and is largely found in bone tissue, as its presence is beneficial to bone strength and growth. Its ion is the fourth most abundant cation in human body with the daily intake of 300-400 mg in the normal adult [61]. Mg is a co-factor for several metabolic enzymes and stabilizes the structure of DNA and RNA [62]. Mg deficiency is reported to cause cell membrane dysfunction, increased incidence of cancer, heart disease and susceptibility to oxidative stress, while excess magnesium level could lead to muscular paralysis, hypotension, respiratory distress and cardiac arrest. However, this is considered be unlikely due to the efficient filtration of kidney [32].

Due to its high amount of daily needs, Mg has been considered to be safe to be fabricated as implantable materials. Mg and its alloys have been utilized in orthopaedic implants due to their supportive physical properties to human bones. Mg has density near to that of the natural bones (1.8-2 g/cm^3) and has been reported to support the activation of bone cells [56]. The only limitation for the Mg for orthopaedic implants is their low corrosion resistance (10-200 mm/year with 99.9% purity in 3% NaCl) [63]. However, the work to enhance the corrosion resistance of Mg have been conducted, including by alloying [64], by coating of dicalcium phosphate dehydrate (DCPD) [65] and by alkali-heat treatment [63].

Nowadays, biocompatibility studies are conducted to test the safety of Mg alloys since pure Mg is known to be tolerable. Aluminium (Al), zinc (Zn), Mn and rare earth elements (RE) such as yttrium (Y) and zirconium (Zr) have been recently reported by Song *et al.* [64] as alloying elements for Mg in order to control its degradation rate. A slower degradation rate of Mg alloys is required in order to give the time to the body to regulate OH^- and H_2 gas which could be generated during degradation. Furthermore, the release of alloying elements such as Al, Mn and Zr might induce toxic effect to the body. Al ions have been reported to induce dementia since it might bind to the inorganic phosphate causing the body lack of phosphate source. Excess Mn also has been reported by Crossgrove *et al.* [66] to cause neurotoxicity that lead to

parkinsinian syndrome. Meanwhile, the presence of Zr is closely associated to the liver, lung, breast and nasopharyngeal cancers [64].

In vitro biocompatibility testing for Mg alloys is conducted in the manner of any other metallic implant materials. Cytotoxicity test using elution medium is often applied as the preliminary toxicity test and followed by the *in vivo* animal implantation. For example, the biocompatibility of binary Mg-Ca alloy was assessed by Li *et al.* [67] using L292 fibroblast cells in which they were exposed to the extract medium of the alloy. The alloy was then fabricated into pins and implanted to the rabbit femoral shaft. The results showed that the Mg alloy pins were completely degraded within 90 days and followed by the formation of new bone tissue. This test was also followed by the plasma serum measurement in which there was no elevated Mg found in rabbit plasma. Thus, the binary Mg-Ca alloy is considered to have a potential use for implant material and further biocompatibility tests are needed in which involving more complex model of implant and animal with regard to the implant functionality and the relevant animal model used to resemble human application.

3.4.2 Iron: State of the art

In the earth crust, Fe is the second most abundant metal element after Al. Fe is an essential element for most of any living organism due to its wide involvement in a large number of Fe containing enzymes and proteins. It plays significant roles in human body which are transport, storage and activation of molecular oxygen, reduction of ribonucleotides and dinitrogen, etc. [68]. It is also involved in the decomposition of lipid, protein and DNA damages due to its reactivity to oxygen molecules which might produce reactive species through Fenton reaction [18].

Pure Fe has a higher elastic modulus (211.4 GPa) compared to that of pure Mg (41 GPa) and its alloys (44 Gpa) and SS316L (190 Gpa) [64, 69]. However, the slow degradation rate (0.16 mm/year) and ferromagnetic nature of pure Fe constitute a problem as implantable devices. Hermawan *et al.* [9] have developed a new Fe-based

alloy which has comparable mechanical properties to that of SS316L and has faster degradation rate (0.44 mm/year) compared to that of pure Fe. In their work, Fe was alloyed with Mn in order to have an austenite phase in which the alloys become antiferromagnetic allowing a good compatibility with the magnetic resonance imaging (MRI), the growing non-invasive diagnostic tools in medical imaging.

In vitro degradation rate of pure Fe also has been confirmed to be promising when Zhu *et al. [70]* showed that *in vitro* degradation elution medium of pure Fe could be beneficial for human endothelial cell proliferation. Human endothelial cells were metabolically inhibited only with elution medium in the concentration higher than 50 μg/ml regardless of incubation time. Furthermore, *in vivo* implantation test of pure Fe has been reported in the field of interventional cardiology. Mesh-like tubular scaffolds (stents) that used to treat atherosclerotic coronary artery were fabricated from pure Fe and implanted in the descending aorta of New Zealand rabbits [71]. Thromboembolic complications, significant neointimal proliferation, systemic toxicity, pronounced inflammatory response was not observed during the study up to 18 months. They have predicted that a single 20 mg cardiovascular Fe stent will be completely degraded after 1 month and the maximum degradation products was considered to give no toxic effect to adjacent tissues. However, *in vivo* degradation kinetic of Fe stent may give a different result to their *in vitro* experiment.

3.5. Biocompatibility testing: from CRMs to BMs

3.5.1 *In vitro* test

In vitro tests provide rapid results in predicting or screening biocompatibility of materials. They allow a great control over the test environments in contrast to *in vivo* tests where animal or human subjects may be influenced by variables such as sex, age, activity, diet, etc. However, it must be noted that *in vitro* test results may not necessarily reflect *in vivo* performance due to the dynamic cell and tissue interactions,

hormonal, and other physiological processes within living organism. Mjor *et al. [72]* have shown that *in vitro* test with zinc-oxide eugenol cement exhibited a severe toxicity, but there was insignificant tissue reaction when used in properly prepared tooth cavities. In this case, dentin in the tooth structure acts as a barrier to reduce concentrations of molecules released from the cement. This study showed an example that *in vitro* cytotoxicity testing should be used only to supplement *in vivo* experiments.

Classical methods such as elution test and direct contact test have been widely used to confirm biocompatibility of corrosion-resistant metals. They are generally followed by viability tests such as trypan blue exclusion test and lactate dehydrogenase (LDH) release. Briefly, cells which are considered viable could pump out the trypan blue out of the cytoplasm thus will appear transparent under the light miscroscope while non-viable cells appear blue. LDH is a cytosolic mammalian enzyme that could leak outside the cell when the membrane integrity is altered, the leakage of this enzyme then quantified based on spectrophotometry measurement of NADH (nicotinamide adenine dinucleotide) production catalyzed by LDH [73]. Cellular proliferation assays based on DNA quantification, metabolic activity measurement (tetrazolium salt-based assays), and cell counting are also commonly conducted following the elution and direct contact tests [74-76]. This assay has been done to conduct the cytotoxicity of Fe [70, 77] as well as Mg [64, 66] both pure or in the form of alloys as mentioned in section 2.

Platelet adhesion and haemolysis tests are also conducted to assess material hemocompatibility for blood-contact implants. Platelet adhesion is one of important steps during blood coagulation, it is conducted to evaluate the antithrombogenicity of biomaterials [78]. Platelet adhesion test on biomaterial is conducted by exposing the materials with platelet-rich plasma followed by scanning electron microscopy (SEM) analysis [79]. Haemolysis test is commonly conducted to evaluate the haemocompatibility of blood-contacting materials. Haemolysis can be induced by the design of the device and material properties such as chemical composition and

physical surface state [80]. Haemolysis test is conducted by measuring the release of haemoglobin from erythrocyte using spectrophotometer [81].

Once the biomaterial implanted, plasma proteins will be absorbed subsequently. This event could trigger the activation of complement system which plays an important role in host defense against infection and foreign bodies. There are at least thirty plasma or cell-bound proteins involved in the system that act as enzymes or simply binding proteins [81]. Biomaterials activate the complement system through the alternative pathway [82], binding protein such as C3b and C4b could bind to particles, surfaces, or bacteria which facilitates the inflammatory cells to recognize foreign bodies [83]. Furthermore, complement system also releases C3a, C4a, and C5a humoral peptides that bind to specific receptors on neutrophils, monocytes, macrophages, mast cells, and smooth muscle cells [84]. This binding causes them to become hyperadherent to the blood vasculature [85]. Neutrophils, which are released from the bone marrow, are the primary phagocytic cells involved in host and foreign body interactions with the ability to migrate, adhere and phagocyte [86]. Once adhered, they can be activated in order to implement their function as phagocytes. The activation of adhered neutrophils is measured by the chemiluminescence assay using 2-methyl-6-phenyl-3,7-dihydroimidazo (1,2-a) pyrazin-3-one hydrochloride (CLA) [87]. By measuring neutrophil adhesion and activation, the inflammatory property of the materials can be estimated.

Furthermore, macrophage test system also used to evaluate the inflammatory reaction towards biomaterials. Macrophages are often used due to their role in immunological system. In early inflammatory reactions, macrophages act as phagocytes cells that engulf the foreign body up to certain sizes. Based on the report by Tabata *et al.* [88], the maximum size of the polystyrene and phenylated polyacrolein microspheres that could be phagocytosed by mouse fibroblasts was in the range of 1-2 μm. Murine macrophages could phagocyte up to 20 μm carbon particles from fibre-reinforced pyrolytic carbon, while larger carbon particles were surrounded by aggregations of macrophages as reported by Brandwood *et al*. [89].

Macrophage activity is morphologically observed under a light microscope after haematoxylin-eosin staining of tissue section as described elsewhere [90]. Qualitative measurement of the presence of macrophage has been reported by Peuster *et al.* [90], in their works, varied levels of macrophage infiltration were observed by haematoxylin-eosin staining on tissue cross section after 18 months implantation of iron stent in rabbit descending aorta. Quantitatively, the phagocystosis activity of macrophages was reported by Greenspan *et al.* [91] by determining the number of internalized latex particles by macrophages. Their works showed that low dose of cadmium (1.5 mg/m^3) exposure increased the phagocytosis activity of rat's alveolar macrophage up to 8 days exposure time.

The classical assessment methods are based on the concern of cell-material physical contact and the probability of released elements. *In vitro* biocompatibility tests for BMs should be conducted in a way that highlight the degradation products toxicity. They can be conducted in the same way that corrosion resistance metals are evaluated, but the release of the degradation products should be already expected. In fact, the release of degradation products is probably the key to assess biocompatibility of BMs. Furthermore, degradation rate and degradation mechanism influence the release of degradation products. These two factors are strongly influenced by the *in vitro* cell culture system. Long-term static cell culture system could indicate a significant toxicity level of BMs due to the accumulation of degradation products within culture medium. This phenomenon may lead to false positive toxicity results and fails the potential materials as reported by Mjor *et al.* [72] when they evaluated zinc oxide eugenol and by Gross *et al.* [92] when evaluating HA bonding ceramic. Thus, the biocompatibility assessment of BMs should be conducted in relatively short period or in a dynamic system in order to anticipate the excessive accumulation of degradation products.

Another potentially interesting *in vitro* technique to assess BMs is by exposing the known degradation products to cell culture. The amount of degradation products as well as the degradation product composition is conducted by another *in vitro* corrosion rate and identification tests. Viability tests, proliferation assays,

hemocompatibility, and inflammatory response are also applicable to be conducted as the endpoint.

3.5.2 *In vivo* tests

In vivo experiment is considered useful since it simulates the real surgical implantation situation. Selection of suitable animal models with regard to the medical devices being tested is important in order to predict the host response in the presence of implant materials. However, *in vivo* experiments also have some disadvantages as they are relatively expensive and laborious compared to that of *in vitro*, therefore they are often suggested after *in vitro* experiments have shown potential advantages of biomaterials.

Carcinogenicity is one of the important *in vivo* assays to test the biocompatibility of materials. Carcinogenicity of metal elements have been reported since 1956 when Heath *et al.* [93] found that 17 out of 30 rats injected with cobalt (Co) powder developed tumour, in which 11 out of 17 are rhabdosarcomas. Other metals known to be carcinogen according to the International Agency for research on Cancer (IARC) are nickel (Ni), chromium (Cr), cadmium (Cd) and arsenic (As). The proposed mechanism to explain this carcinogenicity is associated with reactive oxygen species (ROS) production through the Fenton reaction [94]. In presence of metallic ions, ROS could induce DNA damage, DNA repair inhibition and spindle interference. The alteration of the cell cycle of normal cells could induce uncontrollable proliferation that could lead to the tumour development.

Genotoxicity test is also conducted to confirm the minimal effects of materials to induce gene mutations (base-pair mutations, frame-shift mutation and deletions), chromosomal aberration and DNA defects. This test is needed to show whether the degradation/leachable products of the materials induce genomic alterations. In addition, sensitization test which scores redness and swelling by the materials or their extracts as well as the systemic effect of materials to induce tissue damage are also

commonly conducted to support the evident of biocompatibility.

After all, there is no considerably different technique of *in vivo* tests for BMs [95]. The key parameter is to assess the mechanism of degradation *in vivo* and how can this degradation process induce immune response and toxicity both locally and systematically. Therefore, the carcinogenicity as well as genotoxicity tests are considered appropriate to be conducted to assess the biocompatibility of BMs.

3.6. Assessing biocompatibility by genetic regulation tests

Biocompatibility tests for BMs constitute a new direction with the main challenge related to the released metallic ions as degradation products. The release of metallic ions could lead to imbalance charges in the surrounding tissues and may induce the evolution of reactive species which are closely related to DNA damage and could end up to cell death or could even induce cellular over proliferation [96]. These events of toxicity or carcinogenesis are attributed to altered genetic regulation of the cells due to the presence of degradation products. Hence, genetic regulation plays important roles in dealing with the degradation products to decide the cellular behaviour. This is the key to predict the biocompatibility of BMs.

In a specific case, systemic toxicity caused by degradation products from micro-dimension implants such as cardiovascular stent, is considered to be unlikely due to the dilution effect of the body liquid, liver detoxicification activity and the excretion of kidney. However, the degradation products might induce toxicity to the surrounding cells creating a local toxic effect. This local toxicity effect could be beneficial to inhibit the smooth muscle cell over-proliferation activity that leads to a re-blockage of stented arterial lumen (restenosis). This anti-proliferation effect of degradation products could avoid the employment of anti-proliferation drugs on stent surface which emerges another biocompatibility issue for the drug carriers [18].

From this point, a prospective way has been revealed. Assessing the biocompatibility of degradable materials can be performed through the basic mechanisms of the

cellular response to foreign bodies. The altered cellular behavior driven by their genetic regulation is proposed as an approach to investigate the biocompatibility of BMs. This is the first level detection of biocompatibility since the conventional tests are based on the phenotypic rather than genotypic investigation; more accurate results are then expected through this proposed approach.

Certain gene families have been reported to external disturbances. A situation similar to metal ions exposure. Reports on genes that are related to metal exposures have been recently published [97]. Some of these genes could be candidates as potential biomarkers to assess BM as listed in Table 3.1. Furthermore, suitable gene markers for each parameter are detailed in Table 3.2. These biomarkers constitute key parameters in predicting the cellular behaviour towards the metals and their degradation products.

Table 3.1. Potential gene families to assess biocompatibility of degradable metals [6].

Gene family	Function
Antioxidant	Neutralizing the reactive species
Matrix metalloproteinase	Tissue remodelling
Tumor supressor	Cell cycle control
Cytokines	Chemical inflammatory signals
Oncogenes	Proliferation response

Table 3.2. Gene markers to assess biocompatibility of degradable metals [6].

	Classical method	Gene marker
In vitro		
Cell adhesion	Endothelium adhesion	ICAM-1, VCAM-1
Cell death	Trypan blue	p-53, p-21
Inflammatory response	Neutrophil adhesion	IL-1, IL-6, TNF-α
cell proliferation	Cell counting	TGF, SMGF
In vivo		
Carcinogenicity	Implantation	c-fos, c-myc, c-jun
DNA damage	Chrosmosomal staining	SOD-1
Fibrosis	Immunohistochemistry	COL-α1

3.6.1 Antioxidant genes

BMs used for implantable medical devices are designed to break down into their forming elements until they are fully disappeared. Based on this fact, one proposed idea to assess the biocompatibility of BMs is through the oxidative stress created by the accumulation of metal ions within the cells. Thus, genes that responsible to encounter oxidative stress could be utilized as biomarkers.

Free metal ions which are released during degradation could bind to various metal chelators such as adenosine 5'-diphosphate (ADP), histidine, ethylenediaminetetraacetic acid (EDTA), citrate, etc. They form complexes which catalyze the formation of ROS through the Fenton reaction [98]. In this reaction, superoxide and hydrogen peroxide, which are generated from the partial reduction of molecular oxygen during cellular respiration, will be converted into hydroxyl radicals that are highly reactive [63]. Hydroxyl radicals may initiate lipid peroxidation or undergo reaction with deoxyribosyl back bone of DNA to end up with DNA strand break [95].

ROS could also be generated by metal-catalyzing reaction and the increase of oxygen take up by macrophages, eosinophils and neutrophils during the inflammation periods. Thus, ROS formation can be considered to be an important parameter to be used in assessing the biocompatibility of metallic degradable materials. An index of ROS production could be obtained by measuring the expression of Cu/Zn superoxide dismutase (SOD1), an antioxidant enzyme defence system against ROS. SOD1 exists in two isoforms consisting Cu or Zn in their catalytic center, highly distributed in the cytoplasm, nucleus, peroxisome and inter-membrane space of mitochondria. Elchuri *et al.* [99] have studied the correlation between SOD1 and oxidative damage by knocking down the SOD1 gene in mice, whereas the results showed that the gene was responsible for an increase in oxidative damage and altered hepatic cellular proliferation.

SOD1 has also been reported by Hu *et al.* [97] to participate in the recovery process of cellular oxidative stress. In their work, proteome analysis of *Saccharomyses cerevisiae* was conducted in the presence of various metal salts in order to create oxidative stress conditions. Yang *et al.* [100] utilized SOD1 gene to assess the biocompatibility of four different dental resins, including 2-hydroxyethyl methacrylate (HEMA), bisphenol A-diglycidyl dimethacrylate (Bis-GMA), urethane dimethacrylate (UDMA) and triethyleneglycol dimethacrylate (TEG-DMA). Those materials were tested for their potentials to generate ROS using the SOD1 gene in which green fluorescence protein (GFP) was inserted to the chromosomal copy of *Saccharomyces cerevisiae* as a probe for SOD1 expression. In general, the results showed that SOD1 expression has been successfully used to assess material biocompatibility. TEG-DMA showed an increased expression of SOD1 compared to the other materials and considered to be the least biocompatible.

3.6.2 Matrix metalloproteinase genes

Matrix metalloproteinase (MMP) genes are a family of extracellular matrix (ECM) protein degrading enzymes such as collagens, elastin, laminins and proteoglycans which consist of at least 25 members. The 'metallo' prefix was given since they need the metallic ion Zn^{2+} to catalyze their activity. MMPs involve in a variety of homeostatic functions, such as bone remodelling, wound healing, and several aspects of immunity. They also involve in a number of pathological processes, such as tumour progression, fibrosis, chronic inflammation, tissue destruction, and more [101].

MMPs genes have been used to study the remodelling of human atherosclerotic coronary artery by Pasterkamp *et al*. [102]. In their study, the expression of MMPs genes was detected in the level of protein using monoclonal and polyclonal antibody against each type of MMP genes. The results indicated that MMP2 and MMP9 have

been shown to be more expressed in the expansively remodelled atherosclerotic coronary artery segments. The results have confirmed the function of MMP2 and MMP9 in degrading the collagen type IV as the second prevalent component of arterial ECM, since they were expressed more in the expansive remodelled segment [103].

MMP activity plays an important role to facilitate macrophage mobility towards the production of inflammatory mediators such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) [104]. MMP degrades ECM around the tissue in order to facilitate macrophage movement. Similar function is also needed by vascular smooth muscle cells (VSMCs) when they have to migrate during the morphogenesis of atherosclerotic plaques [105]. This important function of MMP could be used to estimate the activity and severity of body response in the presence of implant and its degradation products.

3.6.3 Tumour suppressor genes

p53 is a tumour suppressor gene leading to cell arrest and apoptosis induced by DNA damage, oncogene, loss of survival signals, microtubule inhibitors, telomere erosion, hypoxia, etc. [106]. p53 induces the expression of p21 gene that will disturb the formation of cyclin-dependent kinase (CDK) and cyclin active complex. This complex is needed in order to promote the progression and cell cycle entry. The presence of p53 gene products will lead to the arrest of cell cycle at the G1-S check point thus allowing DNA repair [107]. The arrested cell cycle can be transient or permanent, if severe DNA damage occurred, the p53 gene will lead to programmed cell death stage or apoptosis [108].

The expression of p53 gene has been reported to be up-regulated in the presence of ferrous ions as the degradation products of Fe implants. The report was done by Mueller *et al.* [18] in which they added Fe(II)-D-gluconate-dihydrate, an Fe supplement, to human smooth muscle cells (SMC) culture and followed by the genetic profiling of SMC using microarray experiment. The expression of p53 genes was in

accordance with the elevated amount of other genes related to the cell death and the decreased expression of metabolic activity and proliferation. However, the p35 gene was not further described in this work. Furthermore, Meplan *et al.* [109] have investigated the effect of Cd on MCF-7 breast cancer cells which express normal level of p53. The results showed that 10-30 mM Cd impaired p53 induction in response to DNA damaging agents such as actinomycin D, methylmethane sulfonate and hydrogen peroxide and suggesting the role of Cd in inducing carcinogenicity.

The use of p53 gene as a marker for assessing the biocompatibility of biomaterials has been reported by Kato *et al*. [110]. They investigated the oncogenes (c-fos and c-myc) and p53 responses of human umbilical cord fibroblasts in presence of various polymers such as tissue culture polystyrene, high-density poly(ethylene), silicone, nylon, tetrafuoroethylene-hexafuoropropylene copolymer, regenerated-cellulose, etc. This study has suggested the efficacy of p53 gene as a powerful method to evaluate the carcinogenicity of biomaterials in which p53 expression was high for the cells which are adhered to hydrophilic surfaces. The biocompatibility of titanium (Ti), NiCr alloy and CoCr alloy was also investigated by Van Kooten *et al.* [111] by studying the expression of p53 gene on human umbilical vein endothelial cells (HUVECs) directly grown to the surface of metals or exposed to the elution medium of each alloy. Results confirming the potential of p53 gene to define cell function in the presence of biomaterials. The expression of p53 gene was increased in the order of Ti, CoCr and NiCr. p53 gene has also been used by Ali *et al.* [112] to assess the genotoxicity of a synthetic biomaterial called polyhydroxybutyrate in the CCL-171 fibroblast cell culture. The results showed a negative genotoxic activity of polyhydroxybutyrate.

3.6.4 Cytokines genes

Cytokines are low molecular weight proteins produced during infection, injury, or in presence of antigens by activated macrophages, monocytes, fibroblasts and endothelial cells [113-115]. Cytokines that promote inflammation are called proinflammatory cytokines, while some that act contrary are called anti-inflammatory cytokines. Interleukin-1 (IL-1) and tumour necrosis factor (TNF) are the principal proinflammatory cytokine for acute phase inflammatory response [114]. IL-1 regulates fibroblast growth, proliferation and protein synthesis which are involved in the destruction of the ECM by stimulating synthesis of collagenase [116, 117]. Similarly, TNF involves in lipid metabolism, coagulation, insulin resistance, and endothelial function [117, 118]. Increased cytokine expression may indicate inflammation or disease progression since they are not detectable in body fluids or tissues under normal condition [115].

The use of IL-1 and TNF-α to assess the biocompatibility of materials has been reported by Miller *et al*. [119]. They have investigated the biocompatibility of biomedical polymers such as polyethylene (PE), silica-free polydimethylsiloxane (PBMS), woven dacron fabric, expanded polytetrafluoroethylene (ePTFE) and the segmented polyurethane (biomer) in combination with lipopolysacaride (LPS) on IL-1 production. In their works, monocytes were cultured on the polymers discs and followed by the measurement of IL-1 expression. They categorized those polymers into three categories based on their reactivity where PBMS and biomer were considered to be low inducer, ePTFE intermediate, and Dacron and PE were considered high. The similar study was also conducted by Cardona *et al.* [120] who investigated polyurethane (PU), ePTFE, woven Dacron and Dacron velour on IL-1 and TNF-α productions using human monocytes cell cultures.

Although measurement of IL-1 and TNF-α in assessing the biocompatibility of BMs has not been developed yet, previously mentioned studies provide evidence for their potentials. Furthermore, the use of anti-inflammatory cytokines could constitute an important approach. IL-4, IL-6, IL-10, IL-11, IL-13, and tumor growth factor (TGF)-

beta are the examples of anti-inflammatory cytokines that could be used. Those cytokines are able to suppress the production of proinflammatory cytokines thus determines the degree of inflammation [121].

3.6.5 Oncogenes

BMs implantations can promote acute response as results from tissue injury during implantation, presence of foreign bodies and the release of metal components. Injuries, foreign bodies and metal ions can induce oxidative stress in the tissue due to the imbalanced charges. Oxidative stress may lead to DNA damage which ends up to genetic mutations causing the development of cancerous cells. The altered genes that promote the cellular over proliferation are called oncogenes. c-fos, c-myc and c-jun are the well-studied genes among the oncogene family.

Huang *et al.* [122] have investigated the biocompatibility of dentin bonding agents using oncogene expressions. The expression level of c-fos and c-jun oncogenes in human gingival fibroblast culture were assayed after being exposed with elution medium. Results showed that all commercial dentin bonding agents induced c-fos and c-jun expression level. Significant increases of expression level were observed during the first hour of incubation period. c-fos was up-regulated transiently since the level of expression was decreased after 3 and 6 hours incubation period. c-jun expression was persistently up-regulated at 1 hour, 3 hour, and 6 hour incubation periods. Furthermore, the cancerous effect of dentin bonding agents was considered due to the release of the resin monomer. The other study conducted by Kato *et al.* [110] showed that human fibroblasts expressed more c-fos when exposed to hydrophobic polymer surfaces such as tissue culture polystyrene, high-density poly(ethylene), nylon, regenerated-cellulose, etc. The results were confirmed with the inverse regulation of p53 when treated with hydrophobic surfaces. These results indicate that oncogene expression could be considered to be potential as markers to assess the BM implants.

However, assessment of BMS by gene markers is inseparable to cell culture experiments. Assessment with gene markers requires RNA (ribonucleic acid) extraction step from the chosen cell lines after exposure to metals and their degradation products. Instead of measuring cell viability and metabolic activity, the endpoint measurement for gene marker experiments could provide further sight of metal exposures towards the cells. Through a quantitative real time PCR (qRT-PCR) technique, the extracted RNA could describe further sight of metal exposures. Not only the activity of the genes that responsible for proliferation could be evaluated, but genes that related to the inflammatory responses, apoptosis (programmed cell death), adhesion molecules, etc. could be analyzed at the same time. Thus the gene markers will show the effect of metals and their degradation products to the exposed cells when compared to control groups.

3.7. Conclusion

The changes in cellular behaviour driven by their genetic regulation are proposed as an approach to investigate the biocompatibility of BMs. With the proposed approach, up- or down-regulated genes that related to the cellular responses due to the presence of degrading metals are used as biomarkers. This approach investigates the biocompatibility at the first level of detection since it measures the mRNA production of the responsible genes for cellular behaviour. Thus, the approach is expected to give accurate information of BMs biocompatibility, where previous conventional *in vitro* test methods are based on phenotypic measurements. Specific gene families that mentioned to be related to tissue remodelling, cell cycle control, inflammatory signals, and ROS-neutralizing activity are expected to support the biocompatibility facts since genetic regulations are the basic mechanism of the cellular function in response to the presence of foreign bodies. This approach is also expected to give advantage for the design and development of metallic materials specific to degradable biomaterial application.

Chapter 4

Gene expression profile of mouse fibroblasts exposed to a biodegradable iron alloy for stents

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4.1 Abstract

Iron based materials could constitute an interesting option for cardiovascular biodegradable stent applications due to their superior ductility compared to their counterparts—magnesium alloys. Since the predicted degradation rate of pure iron is considered slow, manganese (35% w/w), an alloying element for iron, was explored to counteract this problem through the powder metallurgy process (Fe-35Mn). However, manganese presents a high cytotoxic potential, thus its effect on cells must first be established. Here, we explored a new method to investigate biodegradable metals (BMs) by establishing the gene expression profile (GEP) of mouse 3T3 fibroblasts exposed to Fe-35Mn degradation products in order to better understand cell response to potentially cytotoxic BMs. Briefly, 3T3 cells were exposed to degradation products eluting through tissue culture insert filter (3µm pore size) containing cytostatic amounts of 3.25mg/ml of Fe-35Mn powder, 0,25mg/ml of pure Mn powder or 5 mg/ml of pure iron powder for 24 hours. We then conducted a gene expression profiling study from these cells. Exposure of 3T3 cells to Fe-35Mn was associated with the up-regulation of 75 genes and down-regulation of 59 genes, while 126 were up-regulated and 76 down-regulated genes in presence of manganese. No genes were found regulated for the iron powder. When comparing the GEP of 3T3 fibroblasts in presence of Fe-35Mn and Mn, 68 up-regulated and 54 down-regulated genes were common. These results were confirmed by quantitative RT-PCR for a subset of these genes. This GEP study could provide clues about the mechanism behind degradation products effects on cells of the Fe-35Mn alloy and may help in the appraisal of its potential for BM applications.

Keywords: *Degradable metals, stents, fibroblasts, gene expression profiles*

4.2 Résumé

Matériaux à base de fer pourraient constituer une option intéressante pour les applications du stent cardio-vasculaires biodégradables à cause de leur ductilité supérieure par rapport à magnésium. Puisque le taux de dégradation de fer pur est considéré faible, le manganèse (35% w / w), un élément d'alliage de fer, a été étudié pour régler ce problème par le procédé de métallurgie des poudres (Fe-35 Mn). Toutefois, le manganèse présente un fort potentiel cytotoxique, donc son effet sur les cellules doit d'abord être mis en place. Ici, nous avons exploré une nouvelle méthode pour étudier un matériau biodégradable métallique (MB) en établissant le profil d'expression génique (GEP) des 3T3 fibroblastes exposés à des produits de dégradation Fe-35Mn afin de mieux comprendre la réponse des cellules à MB qui est potentiellement cytotoxique. Brièvement, les cellules 3T3 ont été exposés à des produits de dégradation en utilisant l'insert de tissue culture (3 micron de pores) contenant des quantités cytostatiques de 3,25 mg / ml de poudre de Fe-35Mn, 0,25 mg / ml de poudre de Mn pur ou 5 mg / ml de la poudre de fer pur pendant 24 heures. Nous avons ensuite effectué une étude d'expression génétique de ces cellules. L'exposition des cellules 3T3 au Fe-35Mn a été associée à la hausse de 75 gènes et baisse de 59 gènes, alors que 126 étaient augmentés et 76 étaient baissés en présence de manganèse. Il n'y avait pas de gènes qui ont été régulé par la présent du poudre de fer. L'alliage, pour sa part, a augmenté l'expression de 75 gènes (68 en commun avec le Mn) et réduit celle de 59 (54 en commun avec le Mn). Ces résultats suggèrent que certains gènes pourraient constituer de bons marqueurs cellulaires de l'action des métaux métalliques biodegradables sur les tissus mis en contact avec ces matériaux.

4.3 Introduction

Coronary stents have been developed to provide metal scaffolding after angioplasty in order to limit negative remodelling of a stented artery [123]. This growing interest in stenting over the last 25 years is mainly related to their capacity to cover dissections, to decrease the rate of early ischemic complications compared with angioplasty alone, and to reduce the occurrence of in-stent restenosis (ISR) with rate reaching 10-50% in clinical practice [124]. Drug-eluting stents were then designed to reduce ISR rates through local release of anti-proliferative drugs such as sirolimus and paclitaxel, but the late stent thrombosis remained as a significant risk [125].

Despite the effectiveness of the stents, some concerns remain related to their permanency although it is believed that their scaffolding role is temporary. Once healing and re-endothelization of the artery are completed, the need for the stent is less clear and could even induce late adverse effects such as late thrombosis. In addition, anti-platelet therapy has to be maintained chronically because of fear of such life-threatening events. An elegant option to encompass this problem could be to rely on temporary stents that are biodegradable. Such implants limit restenosis, while providing the necessary scaffolding before their erosion, additionally reducing the likelihood of late thrombosis. However, the choice of an appropriate material that satisfies the requirements for both mechanical properties and degradation rate remains as a challenge.

Several materials have been reported as potential biodegradable metals (BMs) for cardiovascular stent application including pure iron [36], Fe-35Mn alloy [9], magnesium alloy [126], and others. For cardiovascular stent application, iron based materials present suitable ductility compared to that of magnesium-based materials. Therefore, our research group has developed an iron-manganese alloy through a powder metallurgical process. It was found that 35% manganese, here after denoted simply as alloy, showed comparable mechanical properties to that of the stainless steel [9].

The elemental choice for the alloy was based on the essential role of iron and manganese within the human body. Iron found in a wide number of Fe-containing enzymes and proteins. Its significant roles include the transport, storage and activation of molecular oxygen, the reduction of ribonucleotides and dinitrogen, decomposition of lipids, proteins as well as DNA, via its reactivity with oxygen molecules [68]. On the other side, the presence of manganese within the alloy could be a potential challenge since the manganese overdose may lead to intoxication and neurotoxicity [127]. However the excess of manganese is not toxic within the cardiovascular system due to the vast counteracting manganese binding proteins within plasma [128]. The maximum daily intake for iron and manganese is 40 mg and 10 mg respectively [61]. Hence, considering the *in vitro* degradation rate of the alloy, 0.44 mmpy [9], and the total weight of a single cardiovascular stent, 50-100 mg, systemic toxicity provoked by a cardiovascular stent caused by the alloy is unlikely [4]. Notably interesting, a localized and transient toxicity achieved by the coronary stent has been viewed as beneficial in order to inhibit ISR after implantation [18]. Recent progression showed that conventional preliminary tests on the alloy have been conducted [127] and showed promising results, however subsequent investigations are needed.

The development of the alloy as BMs for cardiovascular stent application invites a new challenge in the level of biological performance tests. Conventional methods should not be resorted to assess BMs since their unique behavior differs greatly from that of non-degradable materials. The tests can be applied in the same way that corrosion-resistant materials are evaluated, with the anticipated release of degradation products accounted for. Accordingly, experimental tests will have to compensate for the release of metallic ions as degradation products, which could lead to an imbalanced charge in the surrounding tissue. This imbalance could potentially lead the evolution of reactive species which are closely related to DNA damage, resulting in cell death or cellular over-proliferation [95, 96]. These events are the implications of altered genetic regulation due to the presence of degradation products. Conclusively, genetic regulation serves as an interface between the degradation products and cell

behaviour. This is the important key to predict the biological performance of BMs. Alterations of genetic regulation which determine cell behaviour provide an indication of the biological performance of BMs. This approach could provide a more comprehensive understanding between cells and degrading-materials compared to that of conventional tests such as elution test, platelet adhesion test, *etc.*, which most likely based on phenotypic evaluations [129].

Therefore, this work aims to provide a general gene expression profile of mouse fibroblast cells in the presence of the alloy. It is the intention that the modulation of certain groups of genes in the presence of the alloy will best describe the influences of the alloy as a BM. In the near future, certain genes are expected to be closely related to genetic profile changes towards BM exposures. Hence, these so called biomarkers could be identified and used to determine the biological performance of BMs. By this means, we can expect to assess the biological performance of BMs more predictably than if we implemented classical methods. Important biological performance features, such as cytotoxicity, carcinogenicity, inflammatory response, *etc*., could eventually be analyzed by evaluating the genes responsible for each feature.

4.4 Materials and methods

4.4.1 Cell culture

BALB/3T3 mouse fibroblast cells (ATCC number CL-163, Clone A31) were used for this study and cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum (Thermo Scientific, UT) and 1% antibiotic/antimycotic (Thermo Scientific, UT) at 37°C in a humidified incubator at 5% CO_2. For experiments involving cell exposure to the BMs degradation products, cells were plated at a density of 100cells/µl in 24-well cell cultures plates. Cells were left overnight to adhere to the culture plates. Metal powders of pure iron, pure manganese, and the alloy were used. Powders of pure iron and manganese were obtained from GoodFellow Inc. (Oakdale, PA), while powder of the alloy was prepared by mechanical filing and sieving that produced alloy particles less than 75 µm. Powders were chosen in order to create an extreme condition of high surface area in contact with culture medium. Prior to cell exposure, powders were sterilized under UV light for 30 minutes. Various amounts of metal powders in 500µl of culture medium were poured in tissue culture inserts (3.0µm, Corning, NY) and placed above the fibroblasts monolayer. After 24 hours of incubation, the tissue culture inserts were removed and the remaining medium was aspirated. The wells were then rinsed with PBS 1X and the cells were trypsinized and counted on a haemocytometer.

In certain experiments, the cellular metabolic activity was measured as previously described. After the 24-hour incubation the culture mediums, both non-containing and containing metal powders, were removed and 250 µl DMEM with 10% (v/v) WST-1 (Sigma, Oakville, ON, Canada) was added to each well for two hours at 37°C. Finally, 100 µl of medium from each well was transferred into a 96-well plate and a colorimetric measurement was performed on a spectrophotometer at 450 nm.

4.4.2 RNA extraction and array experiment

After a 24-hour incubation period, total RNA was extracted from 6 different replicates for each condition using the Trizol method following supplier's protocol (Invitrogen, Burlington, ON, Canada). Total RNA was purified using an RNA column purification kit (RNeasy Mini Kit, Qiagen, Mississauga, ON, Canada) and subsequently quantified using spectrophotometer. The gene expression analysis was then conducted using Illumina MouseWG-6_V2 bead chip (Illumina Inc., San Diego, CA) at McGill University and Genome Québec Innovation Centre as previously described.

4.4.3 Quantitative RT-PCR

Total Trizol-extracted RNA samples were diluted to 500 ng/µl as described elsewhere. One-microliter of RNA was subsequently converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, ON, Canada). The resulting cDNA was then diluted 10-fold with water prior to amplification (final concentration corresponding to 5 ng/µl of initial RNA). Five microliters of diluted cDNA were amplified (n=2) by qRT-PCR in a Rotor-Gene thermal cycler (Corbett Life Science, Sydney, Australia) using QuantiTect Primer Assays and QuantiFast SYBR Green PCR kits (Qiagen, Valencia, ON, Canada). To correctly judge the efficiency of the amplification reactions, a no-template control was applied to each run which included both a tube of water only as well as a series of three 10-fold dilutions of the representative cDNA. Quantification of gene expression level was based on the -2ΔΔCt method. The mean of threshold cycle (Ct) values of duplicates for each particular gene were then subtracted by the mean Ct value (hence ΔCt) of the control *housekeeping* gene cyclophillin a (PPIA). The difference in the mean Ct values between groups of treatments (Ct) allows for the calculation of the relative levels of expression of particular genes.

Table 4.1. QuantiTect® Primer Assays used in qRT-PCR analysis of gene expression.

Symbol	mRNA	Accession No.	Catalog No.	Amplicon, bp
TFRC	Transferrin receptor	NM_011638	QT00122745	106
GADD45A	Growth arrest and DNA-damage	NM_007836	QT00249655	149
USP18	Ubiquitin specific peptidase 18	NM_011909	QT00167671	136
DDIT3	DNA-damage inducible transcript 3	NM_007837	QT01749748	144
RAB3D	Member RAS oncogene family	NM_031874	QT00158683	89
PARP14	Poly(ADP-ribose) polymerase family, member 14	NM_001039530	QT01771728	91
CXCL12	Chemokine (C-X-C motif) ligand 12	NM_013655	QT00161112	71
MAGED2	Melanoma antigen, family D,2	NM_030700	QT00104755	125
ACAT2	Acetyl-coenzyme A acetyltransferase 2	NM_009338	QT01771441	96
CAV1	Caveolae protein	NM_007616	QT00252007	86
TCF3	Transcription factor 3	NM_001164149	QT00098196	70

4.4.4 Data analysis

Microarray data was analyzed using the FlexArray 1.4.1 software (Genome Québec) as previously described elsewhere [130]. Briefly, genes with a fold change of greater than or equal to 2 and less than or equal to 0.67 were considered to be regulated if *p* value was less than 0.001. For the generation of the GO trees, the lists of regulated genes were imported into the program GoSurfer. A GO category was considered to be regulated if the *p*-value was below 0.05. Only the implicated up and down-regulated genes ranked within the GO categories were imported into the program EASE [131].

4.4.5 Statistical analysis

Other results are presented as mean ± SEM unless specified otherwise. Inter-group comparisons were done using one-way ANOVA and Tukey's post-test. Statistical significance was set at a $p<0.05$. Data and statistical analysis were performed using Graph Pad Prism version 5.01 for Windows, Graph Pad Software (San Diego, CA).

4.5 Results

4.5.1 Determination of effective metal concentrations

In order to study the effects of degradation products on 3T3-fibroblast mouse cell lining (NIH-3T3), cells were exposed to different amounts of metals. The goal was to determine an efficient amount of metal powders that could yield cytostatic conditions after 24 hours of exposure *i.e*. a relatively stable number of viable cells throughout the incubation period. In this study, powdered metals were used in order to gain optimal surface contact, with particles of a maximum diameter size of 75 μm produced and implemented. The degradation products from the metal powder would diffuse through the tissue culture membrane insert and then come in contact with the cells plated in the bottom chamber. As illustrated in Figure 4.1, in presence of degradation products from the alloy (4-6 mg/ml), the total cell count remained unchanged after 24 hours. On the other hand, the relative metabolic activity as estimated by the WST-1 assay was reduced by more than 70% compared to control cells. Degradation products from Mn powder were about 20-times more toxic compared to the alloy with a cytostatic dose of 0.25 mg/ml. As for the iron powder, both the total cell count and the relative metabolic activity were unchanged for a large span of doses.

We thus chose to expose 3T3 fibroblast to 5 mg/ml of the alloy and 0.25 mg/ml of Mn as showed in Figure 4.1 for the gene expression profiling experiments. As for the iron powder, we chose a dose of 3.25 mg/ml corresponding to the 65% iron content in the alloy that gives cytostatic effect on cell number.

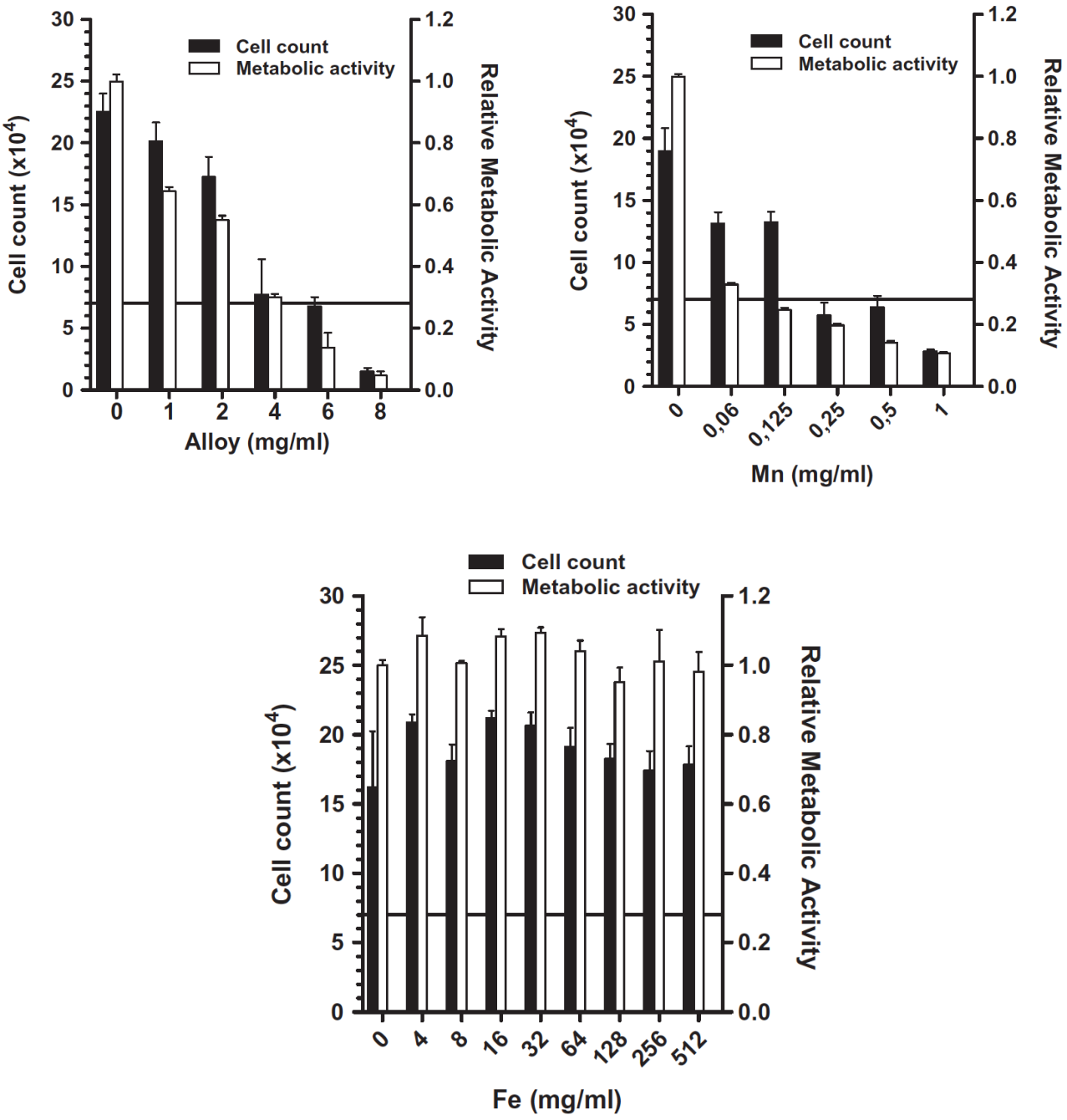


Figure 4.1. Fibroblast cell counting and metabolic activity after 24-hour exposure time with the powdered alloy, Mn, and Fe. The maximum diameter size of powder was 75 µm and the powder was added into the 3µm tissue culture insert. Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Cells were counted under light microscope using haemocytometer and their metabolic activity was measured using 10% WST-1 for 2 hours, the value of the control was set to 1. The bar represents cell count after 24h post-seeding before incubation with BMs.

4.5.2 Gene expression profiling

To investigate the effects of the degradation products from the alloy on the gene expression profile of 3T3 fibroblasts compared to those from manganese and iron powders, the cells were cultured for 24 hours with the amount of metal determined in the previous section. At the end of the experiment, total RNA was extracted, labelled and then hybridized to Illumina MouseWG-6_V2 bead chip. For further analysis, we chose to discard genes displaying low levels of expression. To do so, the mean +5 times the standard deviation of the signals obtained from the DNA chips of genes encoding for olfactory receptors was set as a threshold of minimal expression. The fold change was arbitrarily fixed to levels between treated cells and controls of at least 2 times with a p value less than 0.001.

When exposed to degradation products from the alloy or manganese, larger numbers of genes were regulated. In total, there were 75 up-regulated and 59 down-regulated genes related to the alloy exposure of 3T3 fibroblasts and 126 up-regulated and 76 down-regulated genes related to Mn exposure. No genes were found meeting these criteria when cells were cultured in presence of iron powder.

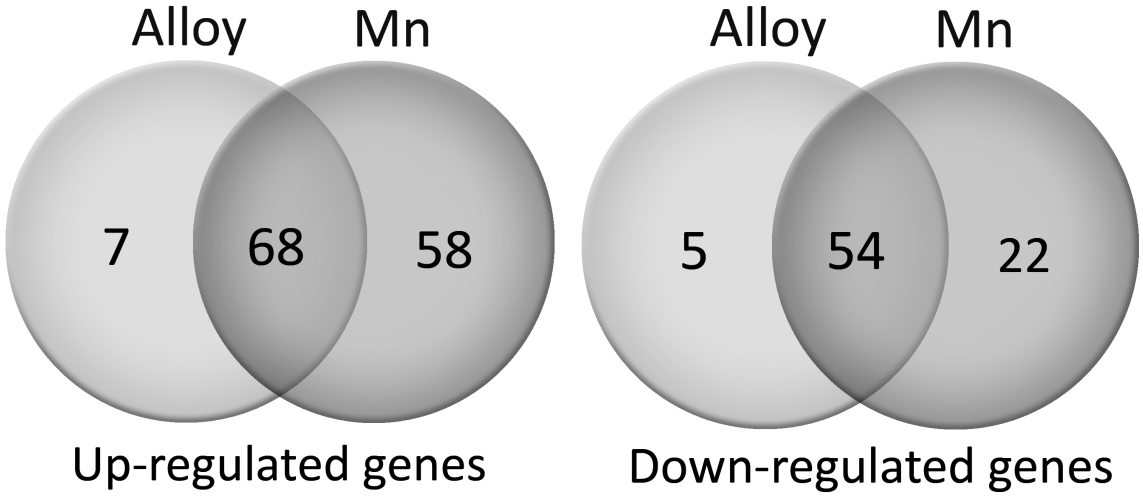
As illustrated in Figure 4.2, 68 up-regulated and 54 down-regulated genes were common between cells exposed to the alloy and manganese powders. Lists of genes that were either up-or down-regulated were then assigned to different processes. As summarized in Table 4.2, when exposed to the alloy, 3T3 fibroblasts up-regulated genes related to coenzyme-related metabolism, followed by carbohydrate-related metabolism. In molecular function systems, electron transport activity was the predominant gene category. As for the down-regulated genes, the presence of alloy predominantly influenced the gene categories related to the foreign bodies and stress response systems in the frame of biological processes. When represented as a hierarchical tree structure, many up-regulated genes in presence of the alloy were related to reactive oxygen metabolism, cholesterol metabolism, *etc*., as depicted in Figure 4.3, while the presence of the alloy provokes fibroblasts response to biotic stimulus, immune response, *etc*. as the most predominant gene categories from the

biological processes system for up-regulated genes (Table 4.3). For down-regulated genes, lipid metabolism, response to foreign body and oxido-reductase activity were amongst the gene categories that were significantly enriched in the presence of the alloy.

Several genes were then chosen from the metal responsive gene groups, especially those that were highly modulated in the presence of metal powders. One of the criteria was also based on the vast existence of the genes throughout the cell lines and species, serving a good candidacy for biomarkers. The chosen genes are listed in Table 4.1. The expression levels of the selected genes were then evaluated by qRT-PCR which simultaneously confirmed the expression patterns obtained from the microarray. The results showed a positive correlation between the microarray data obtained when cells were exposed either to the alloy or the Mn powder and those from qRT-PCR as shown in Figure 4.4 with correlation value (r^2) equals 0,839 and 0,832 for the alloy and manganese, respectively. The expression levels of these genes were then subsequently analyzed for a longer period of 48 hours, allowing for the stability of their expressions. For up-regulated metal responsive genes, DDIT3, TRFC3 and GADD showed a consistent expression level above 2-fold changes at 24 and 48-hour incubation periods, as described in Figure 4.5A. USP18 and PARP14 mRNA levels were strongly reduced after 48 hours compared to 24 hours. As for TCF3, expressions levels observed by microarray did not correlate with those measured by qRT-PCR (Figure 4.4 and 4.5). As for down-regulated metal responsive genes, MAGED2, ACAT2 and CXCL12 were consistently expressed to less than 0.5-fold changes as shown in Figure 4.6. RAB3D and CAV1 showed expression levels higher than expected, notably at 24-hour.

4.6 Discussion

General gene expression profile of mouse fibroblast cells in the presence of the alloy was successfully generated. The resulting gene expression profile is a new approach to study the interaction between cells and materials. Additionally this new approach is considered to be advantageous because it accounts for different degradation behaviour between BMs and their counterparts.



(A) (B)

Figure 4.2. Venn diagram of total number of up-regulated (a) and down-regulated (b) genes exposed with Alloy, Fe, and Mn powders. The diagrams were generated by Flexarray version 1.4.1. When fibroblasts were exposed to alloy, Fe, and Mn, there was no gene that meet the criteria of up-regulated genes (Fold change>2).

In the present study, the effects of a fibroblast cell line exposure to degradation products from iron, manganese and the alloy have been described at the level of gene expression. Local gene expression studies have previously been conducted from permanently-stented pig coronaries by analysing the inflammatory and proliferative responses [90]. Since the data from this study was obtained from RNA extracted from a tissue and only a few genes were studied, it was not possible to have a good portrait

at the impact of the stent on the surrounding cells and their gene expressions. More recently, a gene expression profile of cultured human smooth muscle cells exposed to iron salt (a degradation product of bulk iron) was reported concentrating on the possible effects of degradation products on neighbouring cells [18]. Although the exposed material was not completely comparable to that of the implanted material and its degradation products in use, a significant number of genes required for cell proliferation, cell cycle progression or DNA replication were shown to be down-regulated in the presence of iron salt. This data also suggests a cytotoxicity of ions released from iron stents that could antagonize restenosis by reducing excessive vascular cell proliferation.

Table 4.2. Significant gene category ranking of fibroblasts gene profile exposed to 5 mg/ml alloy powder for 24-hour.

System	Gene Category	List Hits	EASE score
Up-regulated			
Biological Process	coenzyme and prosthetic group metabolism	5	8.8E-4
	biosynthesis	10	2.8E-3
	coenzyme metabolism	4	6.1E-3
	coenzyme biosynthesis	3	1.4E-2
	coenzyme and prosthetic group biosynthesis	3	2.1E-2
	alcohol metabolism	4	2.2E-2
	glucose metabolism	3	2.6E-2
	main pathways of carbohydrate metabolism	3	3.2E-2
	glutamine metabolism	2	3.4E-2
	hexose metabolism	3	3.8E-2
	monosaccharide metabolism	3	4.0E-2
Molecular Function	electron transporter activity	4	2.0E-2
	neutral amino acid transporter activity	2	2.8E-2
	transferase activity	9	3.2E-2
Down-regulated			
Biological Process	response to pest/pathogen/parasite	4	2.2E-2
	isoprenoid biosynthesis	2	2.4E-2
	response to stress	5	3.8E-2
	isoprenoid metabolism	2	4.3E-2
Cellular Component	lipid raft	2	3.0E-2
	Extracellular	11	4.4E-2
	plasma membrane	8	4.8E-2
Molecular Function	structural molecule activity	5	5.0E-2

The list was generated using Expression Analysis Systematic Explorer (EASE) version 2.0
*Number of regulated genes within each category
**EASE probability score, E=exponent, power of 10

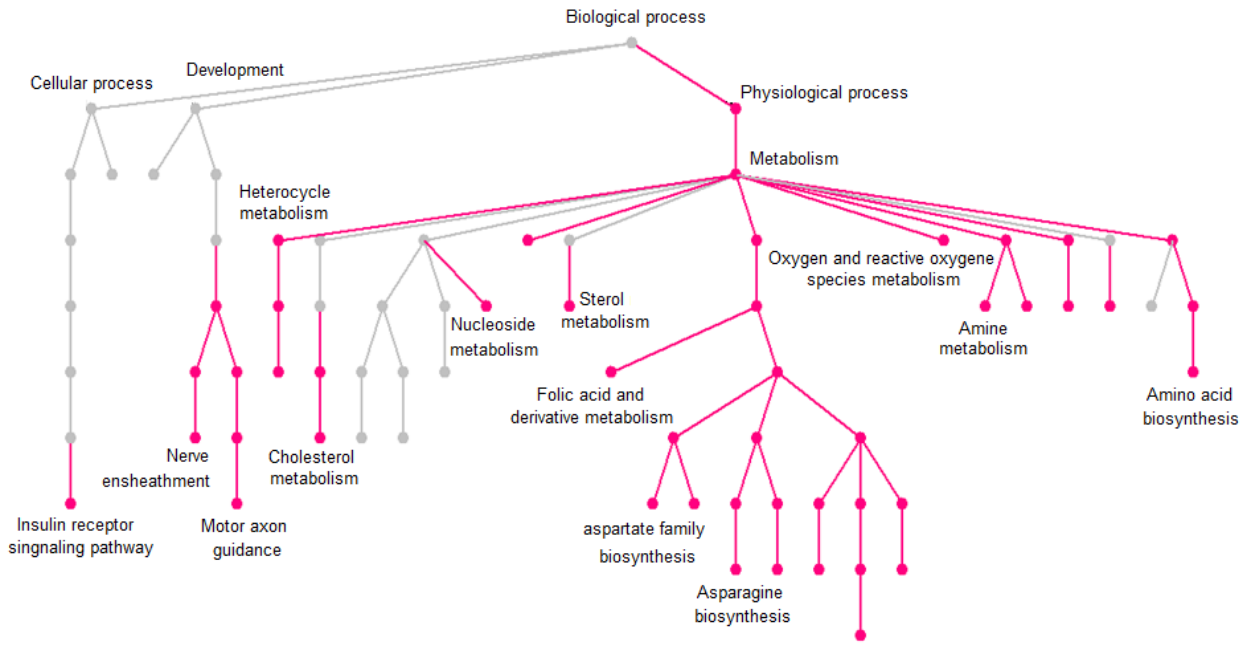


Figure 4.3. Gene ontology tree chart for the up-regulated genes in the presence of alloy powder. The chart was generated with GoSurfer version 1.1 and test for significance was conducted to highlight the nodes that are enriched by genes from the input data (p-value<0,05). Darker nodes on the chart showed the significant nodes.

In vitro model was applied in this study that involves 3T3 fibroblast cells. Fibroblasts were chosen for their implication in extracellular matrix formation, an important feature in neointima formation. In addition, foreign materials, when implanted in an organism, often become encapsulated into collagenous structures and fibroblasts obviously play an active role in this process [132].

Recreating an *in vitro* model that could mimic the complex interaction of a degradable vascular implant is a challenging work. Applying a direct contact assay is often accompanied with significant cell death and limited material to investigate afterwards. We thus chose to expose cells to degradation products diffusing from metal powders that are placed into inserts within a culture medium. Over time, the concentration of degradation products resulting from the metal corrosion will increase and will eventually affecting cells at the bottommost section of the culture

well. In a living artery, these products would first be carried away by active circulation before the implant became encapsulated. Our results show that this encapsulation process could be slowed by the presence of manganese and/or the alloy since the fibroblast expression of various genes associated with extracellular matrix was decreased.

As opposed to previous work by Mueller *et al*. [18], we did not observe significant cytotoxicity for the fibroblasts in presence of iron. At the concentration present in the alloy, iron did not significantly influence the gene expression profile of 3T3 fibroblasts. On the other hand and as expected, manganese degradation products showed marked toxicity despite the relatively low concentration. As reported before, when alloyed with iron, manganese showed decreased cytotoxicity (about 7 times less).

Table 4.3. Significant gene category ranking of fibroblasts gene profile exposed to 0,25 mg/ml Mn powder for 24-hour.

System	Gene Category	List Hits	EASE score
Up-regulated			
Biological Process	response to biotic stimulus	10	4.2E-3
	defense response	8	2.5E-2
	physiological process	51	2.9E-2
	immune response	7	3.1E-2
	biosynthesis	10	3.8E-2
Molecular Function	transcription regulator activity	12	1.2E-2
	transferase activity\, transferring phosphorus-containing groups	9	1.9E-2
	ATP binding	11	2.1E-2
	adenyl nucleotide binding	11	2.3E-2
	purine nucleotide binding	12	3.5E-2
	nucleotide binding	12	3.8E-2
	transferase activity	12	3.9E-2
	Binding	39	4.0E-2
	neutral amino acid transporter activity	2	4.5E-2
Cellular Component	Nucleus	18	4.6E-2
Down-regulated			
Biological Process	isoprenoid biosynthesis	2	3.1E-2
	lipid metabolism	5	3.4E-2
	response to pest/pathogen/parasite	4	3.4E-2
Molecular Function	oxidoreductase activity	6	3.4E-2
	structural molecule activity	6	3.4E-2
Cellular Component	extracellular space	13	2.5E-2
	extracellular	14	2.6E-2
	lipid raft	2	3.9E-2

The list was generated using Expression Analysis Systematic Explorer (EASE) version 2.0

*Number of regulated genes within each category

**EASE probability score, E=exponent, power of 10

Previously, the exposure to iron ions to human smooth muscle cells regulated genes involved in iron homeostasis, DNA replication, and lipid metabolism. Similar results were found when human skeletal muscle cells were exposed to iron, where genes involved in iron homeostasis were found to be considerably regulated. We also observed that some iron homeostasis genes were regulated when exposed to the alloy, although their levels of change in expression did not satisfy the cut-off value fixed to be considered as significant.

While the results of gene expression profile are considered complex, we particularly observed a significant regulation pattern of genes related to carbohydrate metabolism in the presence of the alloy. Little is known about the effect of metal exposures on carbohydrates metabolism, however carbohydrate metabolism genes were previously reported to be regulated in human lung fibroblasts in the presence of heavy metal cadmium and played a role in the activation of protooncogene c-myc [133].

The exposure of the alloy also regulated the expression of genes related to cholesterol metabolism. The same pattern of regulation was shown before in the presence of iron ions as reported by Mueller *et al*. [18]. It has been suggested through an *in vivo* study that metallic elements could decrease the level of cholesterol and ethanolamine phospholipids [134] and could stimulate lipogenesis [135, 136]. Cholesterol is one of key components that determine membrane fluidity. The presence of metallic elements inducing an oxidative stress response could promote an increase in cholesterol metabolism in reaction to membrane instability [18]. Cholesterol metabolism gene category was significantly enriched in the presence of the alloy and manganese alone, but not pure iron alone. This fact implies manganese as an element that closely responsible for cholesterol-related gene regulation.

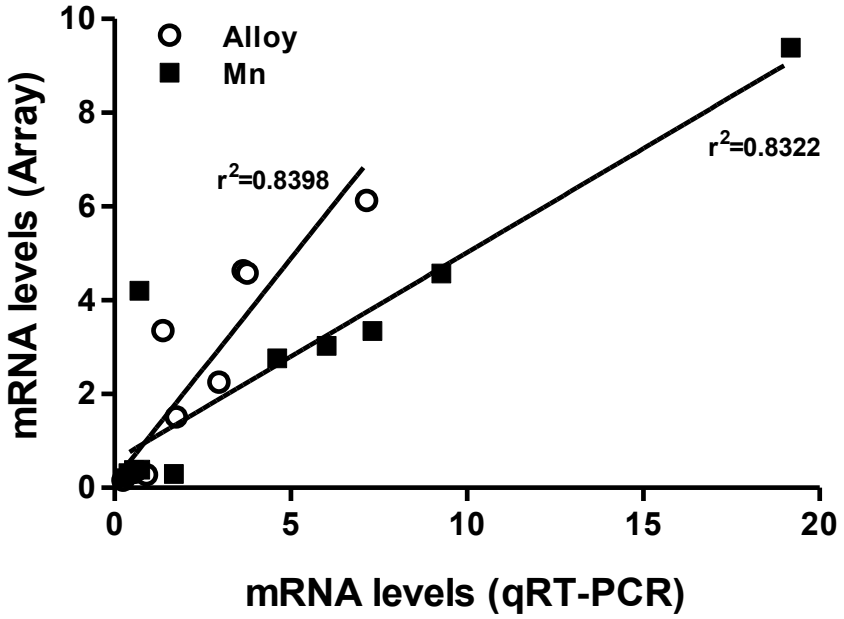


Figure 4.4. Correlation study between microarray expression levels and those of quantitative rt-PCR. Positive correlations were observed on both alloy-influenced and Mn-influenced expression levels with r^2 value of 0,839 and 0,832 respectively. The correlation study was conducted based on the 11-chosen metal responsive-genes and PPIA was used as the house keeping gene standard.

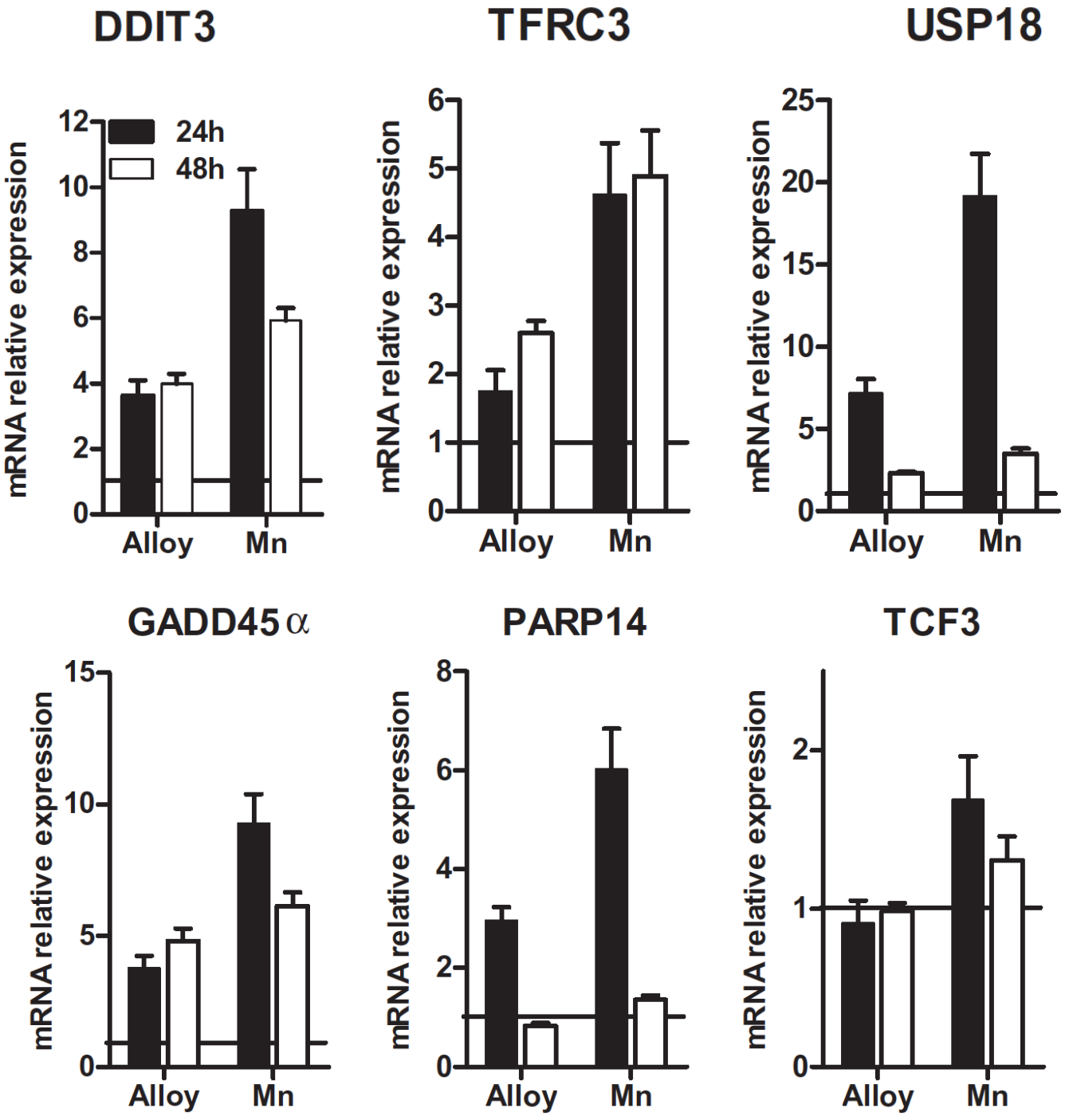


Figure 4.5. Expression level of up-regulated chosen metal responsive-genes at 24 and 48-hour incubation time. The gene expression levels were conducted using quantitative rt-PCR and relative expressions were calculated with regards to that of PPIA as the house keeping gene.

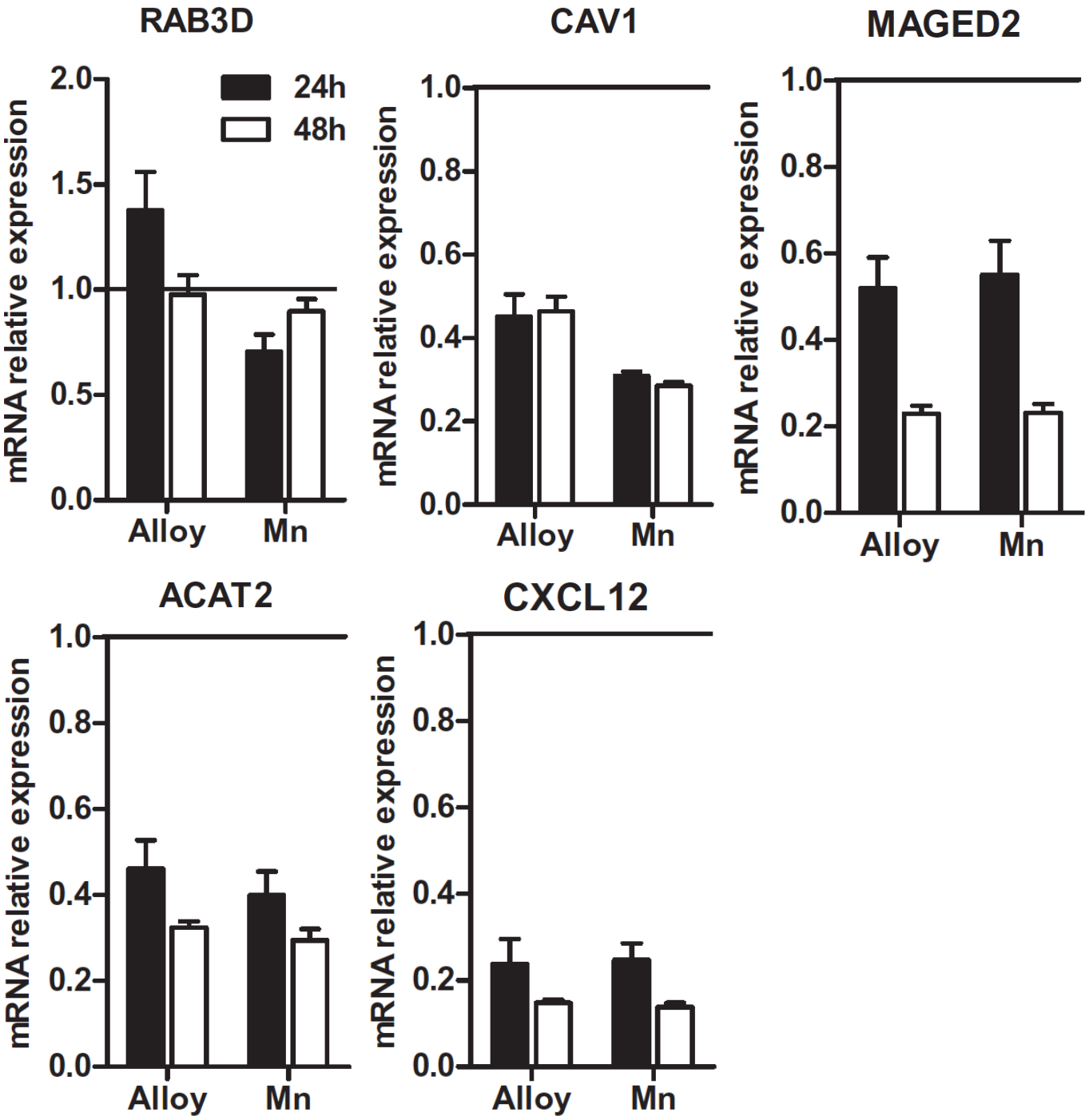


Figure 4.6. Expression level of down-regulated chosen metal responsive-genes at 24 and 48-hour incubation time. The gene expression levels were conducted using quantitative rt-PCR and relative expressions were calculated with regards to that of

PPIA as the house keeping gene.

Moreover, regulation of genes that related to the cell cycle was observed. It was found that the expression pattern of growth arrest and DNA-damage-inducible 45 alpha (Gadd45α) was one of the most significantly up-regulated genes in the presence of BMs, shown in Figure 4.5. Gadd45α is induced by p53, a tumour suppressor gene, leading to cell growth arrest and apoptosis [106]. The induction of Gadd45α could alter the formation of cyclin-dependent kinase (CDK) and cyclin active complex needed to promote cell cycle progression [18]. This finding suggests the effect of BMs exposure to the cell cycle of 3T3 fibroblast. However, more comprehensive gene expression studies are required in order to have a clear idea of BMs exposures towards the alteration of fibroblasts cell cycle. Additionally, our data showed that some selected genes including Gadd45α, showed a stable expression up to 48-hour treatments, making them potentially interesting candidates for biomarkers. Other genes with stable expression pattern towards alloy and manganese exposures are DDDIT3, TFRC3, TCF3, CAV1, MAGED2, ACAT2, and CXCL12. In the future, it will be of importance to confirm these observations made in fibroblasts in other cell types such as endothelial and smooth muscle cells.

4.7 Conclusion

Having the same given proportion within the alloy, pure iron did not significantly influence the gene expression profile of 3T3 fibroblasts. On the other hand, manganese showed a marked cytotoxicity at relatively low concentrations. Some iron homeostasis genes were regulated when exposed to the alloy, although their levels of change in expression did not satisfy the cut-off value fixed to be considered as significant. It was notably noticed that the exposure to the alloy regulates cholesterol metabolism since cholesterol is of the key components that determines membrane fluidity. Exposing cells to a degradable alloy could create membrane instability due to the oxidative stress induced by the released metallic ions. Since the effect made by the alloy was also observed in the manganese exposure, it suggests that manganese, as a component of the alloy, was responsible for this result. At the gene expression level, GADD45α, DDIT3, TFRC3, TCF3, CAV1, MAGED2, ACAT2, and CXCL12 showed a stable expression pattern up to 48 hours, suggesting the need for further study to confirm them as potential biomarkers for BMs.

Chapter 5

Caveolin: a possible biomarker of degradable metallic materials toxicity on vascular cells

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5.1 Abstract

Iron-based materials could constitute an interesting option for cardiovascular biodegradable stent application due to their appropriate ductility compared to their counterparts namely, magnesium alloys. However, the predicted degradation rate of pure iron is considered too slow for such application. We explored manganese (35% w/w) as an alloying element in combination with iron to circumvent this problem through powder metallurgical process (Fe-35Mn). Manganese, on the other hand, is highly cytotoxic. We recently explored a new method to better characterize the safety of biodegradable metals (BMs) by establishing the gene expression profile (GEP) of cells (mouse 3T3 fibroblasts) exposed to Fe-35Mn degradation products in order to better understand their global response to potentially cytotoxic BMs. We identified a number of up- and down-regulated genes and confirmed the regulation of a subset of them by quantative RT-PCR. Caveolin-1 (cav1), the structural protein of caveolae little plasma membrane smooth invaginations present in various differentiated cell types, was one of the most down-regulated genes in our GEPs. In the present study, we further studied the potential of this 22kDa protein to become a biomarker for cytotoxicity towards the exposure of degradable metallic elements. In order to better characterize cav1 expression in this context, 3T3 mouse fibroblasts were exposed to either ferrous and manganese ions at a cytostatic concentrations for 24 or 48 hours. Cav1 gene expression was not influenced by exposition to ferrous ions. On the other hand, manganese exposure for 24 hours reduced cav1 gene expression by about 30% and >65% at 48 hours compared to control 3T3 cells. Cav1 cellular protein content was also reduced to the same extent. The same pattern of expression for cav3 (the muscle specific caveolin subtype) was also observed in the study. This strong and reproducible pattern of regulation of caveolins thus exhibits a potential as a biomarker for the toxicity of BM elements. This type of response to exposure to BMs is similar in both caveolin-1 and caveolin-3 expressing cell types such as endothelial, fibroblastic and smooth muscle cells.

Keywords: Degradable metals, fibroblasts, caveolin

5.2 Résumé

Des matériaux à base de fer pourraient constituer une option intéressante pour l'application stent cardio-vasculaire biodégradable à cause de leur ductilité appropriée par rapport à magnésium. Toutefois, le taux de dégradation prévue de fer pur est considéré trop lent pour une telle application. Nous avons exploré le manganèse (35% w/w) comme élément d'alliage en combinaison avec le fer pour contourner ce problème à travers le processus de métallurgie des poudres (Fe-35Mn). Manganèse, d'autre part, est très cytotoxique. Nous avons récemment exploré une nouvelle méthode pour mieux caractériser la biocompatibilité des métaux biodégradables (MBs) en établissant le profil d'expression génique (GEP) de cellules (fibroblastes 3T3) exposés à des produits de dégradation Fe-35Mn afin de mieux comprendre leur réponse globale au MB potentiellement cytotoxique. Nous avons identifié un certain nombre de gènes régulés et a confirmé la régulation d'un sous-ensemble d'entre eux par RT-PCR quantitative. La cavéoline-1 (cav1), la protéine structurale de cavéoles—petites invaginations de la membrane plasmatique présentes dans divers types de cellules différenciées, était l'un des plus régulés dans nos GEP. Dans la présente étude, nous avons encore étudié le potentiel de cette protéine de 22 kDa comme biomarqueur de la cytotoxicité de MBs. Afin de mieux caractériser cav1 expression dans ce contexte, des fibroblastes de souris 3T3 ont été exposés soit à des ions ferreux et du manganèse à une concentration cytostatiques pendant 24 ou 48 heures. L'expression de cav1 n'a pas été influencée par l'exposition à des ions ferreux. D'autre part, l'exposition de manganèse pendant 24 heures réduit l'expression cav1 d'environ 30% et > 65% à 48 heures par rapport au contrôle. La teneur protéines cellulaires de cav1 a également été réduite dans la même mesure. Le même schéma d'expression pour cav3 (le sous-type cavéoline specifique pour des cellules muscles) a également été observée dans l'étude. Cette l'expression des cavéolines présente donc un potentiel en tant que biomarqueur de la toxicité des éléments MBs. Ce type de réponse à l'exposition des MBs est similaire pour les deux cav1 et la cav3 chez les cellules musculaires, endothéliales, et fibroblastes.

5.3 Introduction

Caveolae, little caves, were first described as flask-shaped plasma membrane invaginations capable of transporting molecules across the endothelial barrier. They are found abundantly mostly in highly differentiated cell types such as endothelial and smooth muscle cells [137]. Caveolae have been associated with a number of cellular functions or processes such as potocytosis, cholesterol homeostasis, transformation, and the control of signal transduction [138, 139]. Their functions are related to their structure, rich in cholesterol, spingomyelin and glycospingolipids. Caveolins, the biochemical marker for caveolae structures also play a central structural role in vesicle formations. The caveolin family comprises at least three genes that are expressed heterogeneously in different cell types. Caveolin-3 (cav3) is specifically expressed in muscle cells [140, 141], while caveolin-1 (cav1) and caveolin-2 (cav2) are expressed in most cell types such as fibroblasts, adipocytes, endothelial cells, and pneumocytes [142]. The implantation of a metallic stent in an artery is associated with a mechanical disruption of the endothelial layer. Using biodegradable metallic stents could also exert additional damages to the surrounding tissue via the release of degradation endproducts. Caveolin 1 has recently been reported as a potential biomarker of vascular injury caused by certain vasodilators [143]. Cav1 is highly expressed in normal blood vessels and its expression is decreased at the site of damage in vascular injury. This raises the possibility that cav1 expression could act eventually as a marker of vascular injury at the site of implantation of a stent. Cav1 also acts as a binding site of nitric oxide synthase (NOS) which is responsible for nitric oxide production [144].

We recently reported that caveolin-1 expression was decreased in a cultured fibroblastic cell line when exposed to the degradation products of biodegradable metals (BMs). In fact, earlier experiments with 3T3 fibroblast cells showed that cav1 was down-regulated in the presence of BMS and that this pattern of expression was observed as early as 24-hour exposures with Fe-35Mn alloy and was stable up to 48-hour exposures. It is generally believed that the presence of BMs is associated to the release of metallic ions that act as oxidants and lead to an imbalanced charge within

the surrounding tissue [6]. Our findings showed that the expression of cav1 was decreased in the presence of BMs which is in accordance to other reported results where ozone was applied as an oxidant in lung-injury mouse model [145]. In this study, we studied further the expression pattern of caveolins when cells are exposed to BMs.

5.4 Materials and methods

5.4.1 Cell culture

BALB/3T3 mouse fibroblast cells (ATCC number CL-163, Clone A31) were cultured in Dulbecco‟s Modified Eagle‟s Medium (Invitrogen, Burlington, Canada) supplemented with 10% fetal bovine serum (Thermo Scientific, UT) and 1% antibiotic/antimycotic (Thermo Scientific, UT) at 37°C in a humidified incubator at 5% CO_2. A10 (ATCC number CL-163, Clone A31) rat smooth muscle cells, SMCs, were cultured in M199 culture medium (Invitrogen, Burlington, Canada) in the same incubation condition to that of fibroblasts. For experiments involving cell exposure to the BMs degradation products, cells were plated in 24-well cell cultures plates at a density of 100cells/µl and 60cells/ µl for fibroblasts and SMCs respectively. Cells were then left overnight to adhere to the culture plates.

Metal powders of pure iron, pure manganese, and the alloy were used. Powders of pure iron and manganese were obtained from GoodFellow Inc. (Oakdale, PA), while powder of the alloy was prepared by mechanical filing and sieving that produced alloy particles less than 75 µm. Powders were chosen in order to create an optimum condition of high surface area in contact with culture medium. Various amounts of metal powders in 500µl of culture medium were poured in tissue culture inserts (3.0µm, Corning, NY) and placed above the fibroblasts monolayer. After 24 hours of incubation, the tissue culture inserts were removed and the remaining medium was aspirated. The wells were then rinsed with PBS 1X and the cells were trypsinized and counted using haemocytometer. In experiments using salts of iron ($FeCl_2$) and

manganese ($MnCl_2$), the salts were directly solubilised into cell culture medium without the utilisation of culture inserts. At the end of incubation period, the cells were counted in the same manner as described above.

In certain experiments, the cellular metabolic activity was measured as previously described [6]. After the 24-hour incubation the culture mediums, both non-containing and containing metal powders, were removed and 250 µl DMEM with 10% (v/v) WST-1 (Sigma, Oakville, ON, Canada) was added to each well for two hours at 37°C. Finally, 100 µl of medium from each well was transferred into a 96-well plate and a colorimetric measurement was performed on a spectrophotometer at 450 nm.

5.4.2 RNA extraction and Quantitative RT-PCR

Total RNA was extracted from 6 different replicates for each condition using Trizol following supplier‟s protocol (Invitrogen, Burlington, ON, Canada). Total RNA samples were diluted to 500 ng/µl as described elsewhere. One-microliter of RNA was subsequently converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, ON, Canada). The resulting cDNA was then diluted 10-fold with water prior to amplification (final concentration corresponding to 5 ng/µl of initial RNA). Five microliters of diluted cDNA were amplified (n=2) by qRT-PCR in a Rotor-Gene thermal cycler (Corbett Life Science, Sydney, Australia) using QuantiTect Primer Assays and QuantiFast SYBR Green PCR kits (Qiagen, Valencia, ON, Canada). To correctly judge the efficiency of the amplification reactions, a no-template control was applied to each run which included both a tube of water only as well as a series of three 10-fold dilutions of the representative cDNA. Quantification of gene expression level was based on the -2ΔΔCt method. The mean of threshold cycle (Ct) values of duplicates for each particular gene were then subtracted by the mean Ct value (hence ΔCt) of the control housekeeping gene cyclophillin a (PPIA). The difference in the mean Ct values between groups of treatments (Ct) allows for the calculation of the relative levels of expression of particular genes.

5.4.3 Western blotting

Cultured cells were collected using trypsin/EDTA and were pelleted by centrifugation. The pellet was then homogenized in the following lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 20 mM β-glycerophosphate, 1% NP-40, 10 mM NaF, 2 mM Na3VO4 and a cocktail of protease inhibitors) and was subsequently transferred to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Moreover, the gel was blotted to nitrocellulose membranes, blocked for 1 h and then incubated for overnight at 4°C with primary antibodies cav1 or cav3.

5.4.4 Statistical analysis

Other results are presented as mean ± SEM unless specified otherwise. Inter-group comparisons were done using one-way ANOVA and Tukey's post-test. Statistical significance was set at a $p<0.05$. Data and statistical analysis were performed using Graph Pad Prism version 5.01 for Windows, Graph Pad Software (San Diego, CA).

5.5 Results

5.5.1 BMs down-regulate caveolin expression in 3T3 fibroblasts.

We chose to conduct the experiments described here at a "cytostatic" dose of a given material or salt. We defined a cytostatic dose as sufficient to affect the proliferative behavior of the cells and to avoid massive cellular death impairing the capacity to perform expression studies. In practice, we performed our incubation at a dose of materials where after 24 hours, the total viable cell number was equal to the one put in culture at the beginning. Previous studies showed that the cytostatic dose of the Fe35Mn alloy (alloy) and manganese in the form of powder is 5 mg/ml and 0.25 mg/ml respectively. As for the iron powder, up to 32 mg/ml did not give any significant effect to 3T3 fibroblast cells. As illustrated in Figure 5.1, caveolin-1 (cav1) mRNA levels were strongly down regulated in presence of manganese powder or the alloy after 24 hours. Iron powder (32 mg/ml) did not changes cav1 gene expression. A similar pattern was observed after 48 hours of incubation. We then checked if the total cav1 protein content followed the same type of regulation as for the mRNA in 3T3 fibroblasts. As illustrated in Figure 5.2, the results obtained by immunoblotting using a specific cav1 antibody on total cellular homogenates separated on SDS-PAGE, were comparable to those of cav1 gene expression. We were not able to achieve a cytostatic concentration using iron powder, we used $FeCl_2$ and $MnCl_2$ salts. The salts are soluble within the cell culture medium providing free metallic ions. These experiments were thus conducted without culture inserts. As shown in Figure 3, following a 24-hour incubation period, we determined that a concentration of 1.5 mg/ml of $FeCl_2$ or 0.025 mg/ml of $MnCl_2$ achieved the desired cytostatic effect in 3T3 fibroblasts. This time, when treated with this cytostatic concentration of FeCl2, caveolin-1 mRNA levels (Fig. 5.4) and protein content (Fig. 5.5) were both decreased similarly to those treated with the manganese salt.

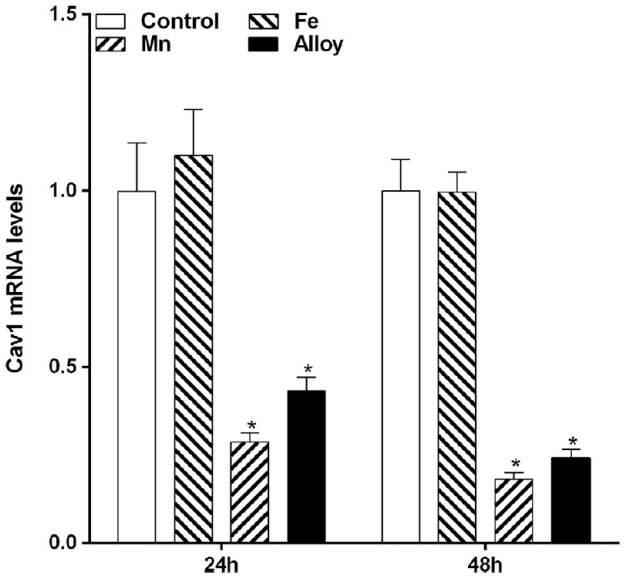

Figure 5.1. Caveolin-1 (cav1) mRNA levels in 3T3 fibroblasts after a 24- or a 48-hour exposure to powdered alloy, Mn, and Fe. The maximum diameter size of powder was 75 μm and the powder was added into the 0,2 μm tissue culture insert. Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to mRNA levels measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control.

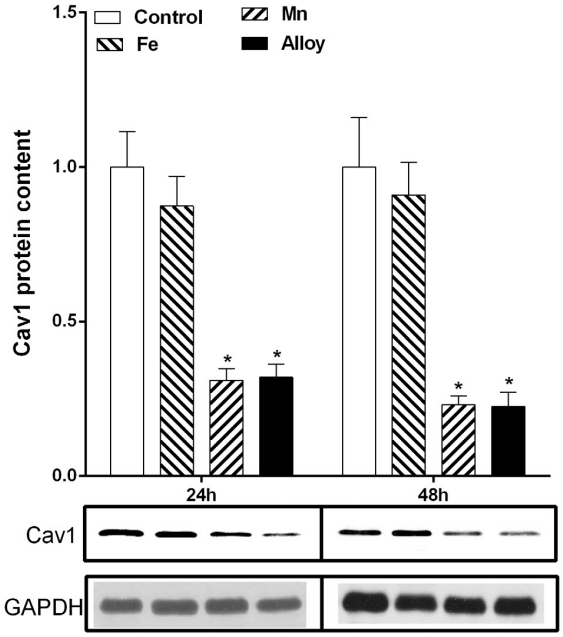

Figure 5.2. Caveolin-1 (cav1) protein content in 3T3 fibroblasts after a 24- or a 48-hour exposure to powdered alloy, Mn, and Fe. Results are expressed as mean ± SEM (n=6) relative to protein expression levels measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of representative results from the cav1 immunoblot for each treatment group is illustrated at the bottom of the graph. The 22 kDa unique band can be seen.

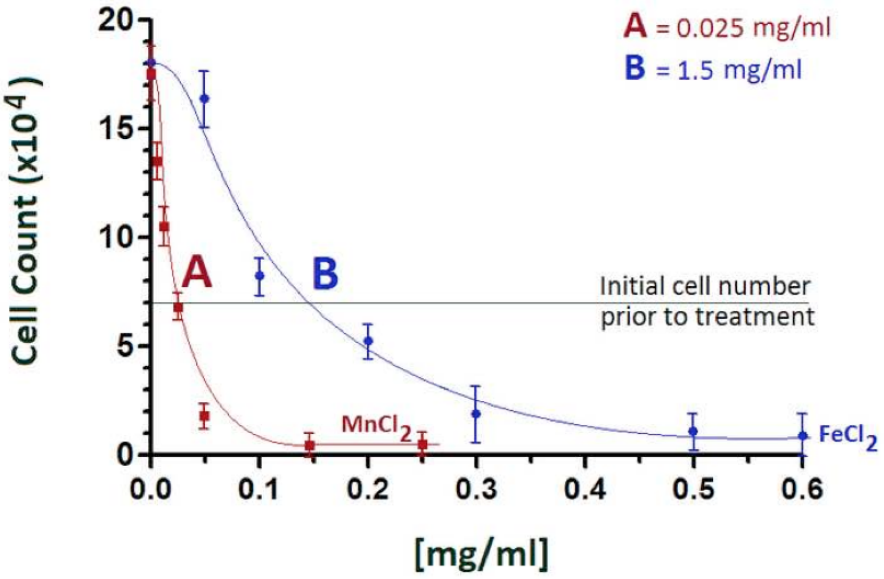

Figure 5.3. Fibroblast cell count after a 24-hour exposure time with either increasing concentrations of $FeCl_2$ or $MnCl_2$. Fibroblasts were pre-cultured for an overnight incubation period prior to metal salt exposures. Cells were counted under light microscope using haemocytometer.

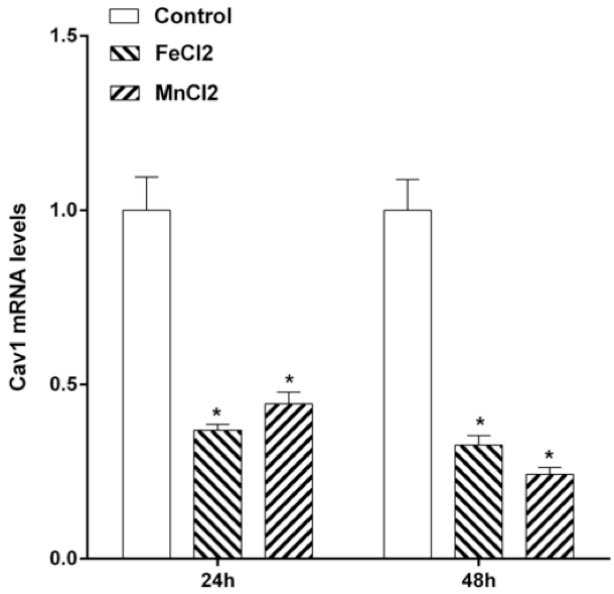

Figure 5.4. Caveolin-1 (cav1) mRNA levels in 3T3 fibroblasts after a 24- or a 48-hour exposure to $FeCl_2$ or $MnCl_2$. Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to mRNA levels measured in control (arbitrarily fixed at 1) *: $p<0.001$ vs. control.

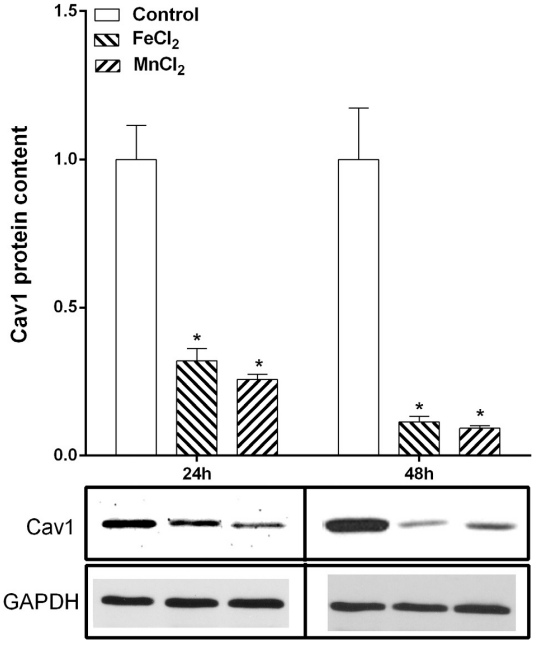

Figure 5.5. Caveolin-1 (cav1) protein content in 3T3 fibroblasts after a 24- or a 48-hour exposure to $FeCl_2$ or $MnCl_2$. Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to protein expression levels measured in control (arbitrarily fixed at 1) *: $p<0.001$ vs. control. A view of representative results from the cav1 immunoblot for each treatment group is illustrated at the bottom of the graph. The 22 kDa unique band can be seen.

5.5.2 BMs down-regulate caveolin expression in A10 smooth muscle cell line.

The main artery wall constituent responsible for restenosis after stenting is the smooth muscle cell. Although smooth muscle cells express a certain amount of caveolin-1, the main caveolin is isoform 3. We used A10 cells, derived from the thoracic aorta of embryonic rat and a commonly used model of vascular smooth muscle cells. As for the fibroblasts, exposition to A10 cells to metal powder reduced drastically caveolin3 protein content (Fig. 5.6). We determined that concentrations as small of 0.1 mg/ml of $FeCl_2$ and 0.05 mg/ml of $MnCl_2$ gave a cytostatic effect to A10 cells (not shown). At these concentrations, total protein content of caveolin-3 was decreased by more than 50% compared to controls (Fig. 5.7). We then tested if the effects of metal salts influences the expression of the alpha smooth muscle actin (α-SMA), a marker for smooth muscle cell. After 48-hour exposure to metal salts, the expression of α-SMA remained unchanged (Figure 5.8).

5.6 Discussion

Our results showed that both cav1 and cav3 was down regulated in the presence of metal elements both in the forms of powder or salt. BMs have been proposed for some specific biomaterial applications, including paediatric, orthopaedic and cardiovascular applications. BMs are designed to disappear via corrosion once the structural support their providing is no longer necessary. Once implanted, this new class of biomaterials is expected to support the healing process of a diseased tissue or organ while degrading at a potentially adjustable degradation rate. We proposed recently that studying gene regulation of cells or a tissue in the presence of BMs could provide interesting insights. In addition to predict cell behaviour in the presence of degradation products, it would be possible to describe the mechanisms behind this response [6]. In addition, determining gene profile of cell responses towards BMs could help identify potential gene products that could serve as biomarkers. The caveolin gene family seems to have the potential to become such biomarker. It shows a strong and reproducible down-regulation in presence of metals both at the level of gene expression and protein content. It is thus possible to envision studying tissues after implantation and evaluate caveolins expression.

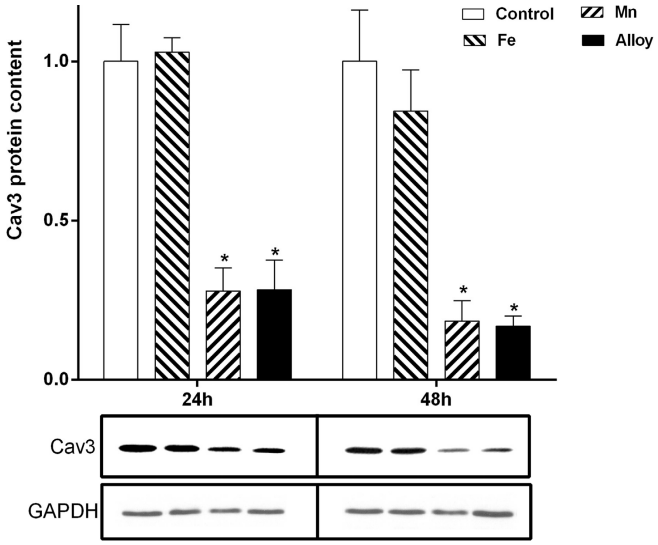

Figure 5.6. Caveolin-3 (cav3) protein content in A10 smooth muscle cell line after a 24- or a 48-hour exposure to powdered alloy, Mn, and Fe. The maximum diameter size of powder was 75 µm and the powder was added into the 0,2 µm tissue culture insert. Fibroblasts were pre-cultured for an overnight incubation period prior to metal exposures. Results are expressed as mean ± SEM (n=6) relative to protein expression levels measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of representative results from the cav1 immunoblot for each treatment group is illustrated at the bottom of the graph.

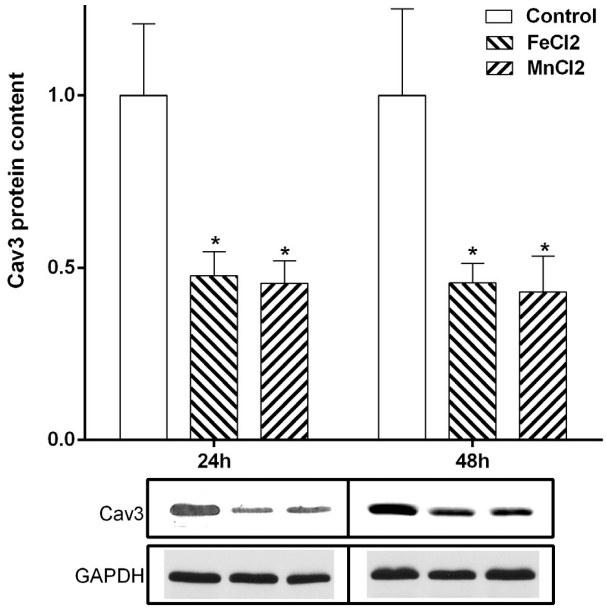

Figure 5.7. Caveolin-3 (cav3) protein content in in A10 smooth muscle cell line after a 24- or a 48-hour exposure to $FeCl_2$ or $MnCl_2$. Results are expressed as mean ± SEM (n=6) relative to protein expression levels measured in control (arbitrarily fixed at 1) *: p<0.001 vs. control. A view of representative results from the cav1 immunoblot for each treatment group is illustrated at the bottom of the graph.

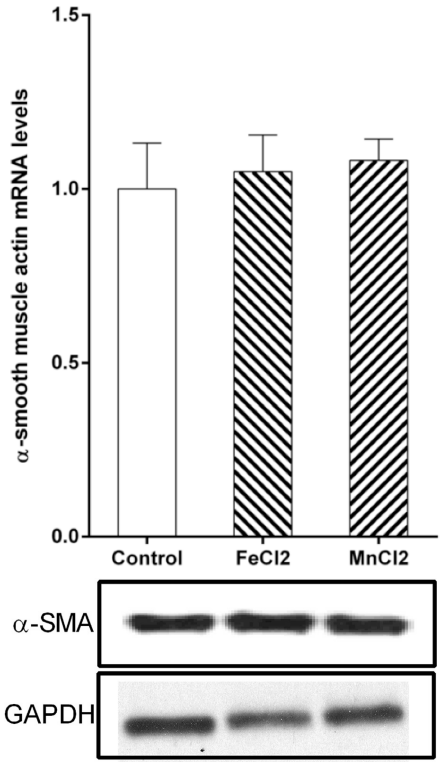

Figure 5.8. Smooth muscle actin alpha (α-SMA) protein content in in A10 smooth muscle cell line after a 24- or a 48-hour exposure to $FeCl_2$ or $MnCl_2$. Results are expressed as mean ± SEM (n=6) relative to mRNA levels measured in control (arbitrarily fixed at 1) *: $p<0.001$ vs. control. A view of representative results from the cav1 immunoblot for each treatment group is illustrated at the bottom of the graph.

Cav1 expression is regulated transcriptionally or post translationally. The cav1 promoter region includes three G+C-rich sites which could act as sterol regulatory elements. Additionally, the promoter region contains a CAAT sequence and a Sp1 consensus sequence [146]. SRE-like elements are involved in the response to stimuli, such as lowdensity lipoprotein-free cholesterol (LDL-FC) [147, 148]. Other transcription factors reported to regulate cav1 expression include the forkhead (FKHR) family of transcription factors, FOXO3a [149], C-myc [147] and NF-κB [150]. Several studies have implicated caveolins during the cellular response to a toxic stress. Endothelial cell caveolin-1 expression is down-regulated after exposure to nanoparticles. Since caveolin controls endothelial nitric oxide synthase, a decrease in cav1 expression could lead to increase NO production and toxicity for the vascular wall [151]. Caveolin-1 levels are also reduced in endothelial cells exposed to cadmium

[152]. On the other hand, treatment of cancer cells with cytotoxic agents (antineoplastic) usually increases caveolin expression [153-155]. Expression of caveolin is down-regulated in many cancer cells [156] lines while highly differentiated cells usually express high level of caveolin [157, 158]. In this study, we exposed fibroblasts and smooth muscle cells which have high levels of caveolins to BM at moderately toxic concentrations and observed a loss of caveolins. Since caveolins are the principal structural protein of caveolae which both acts on plasma transport and signalling [157, 159], this down-regulation may be a way for the cells to protect themselves from the soluble metal oxides or metal ions present in the extracellular space by blocking the endocytosis capacity of caveolae. Interestingly, the expression of cav3 was also down regulated following metal powder and metal salt exposures, the same pattern as cav1 showed. Although the role of cav3 in signal transduction pathways towards cell survival is not well investigated, it has been reported to play a role in the phenotype and the death of cultured muscle cells [158]. In this study, the disruption of caveolin expression was closely related to the presence of metallic elements in the cell culture medium. Excessive presence of metallic elements leads to a high oxidative stress for the cell and discarding caveolae may help the cell to preserve its integrity.

The implantation of a vascular stent is usually made in a region of an artery where atherosclerotic lesions are present. Restenosis is major problem related to stent implantation and this phenomenon is characterized by smooth muscle cell proliferation and increased artery wall fibrosis. The current use of drug-coated stent is aimed at avoiding this unwanted reaction to the stent interaction to the artery wall. Our present work here supports that the degradation of a stent made from BMs could lead to a regional cell proliferation inhibition which could also helped to slow the restenosis process. It will be interesting in the future to test if the effects of BMs on a real artery lead to caveolin down-regulation, a sign of reduced cellular proliferation.

5.7 Conclusion

The mRNA expression study showed that cav1 was constantly down regulated in the presence of metallic elements both in the form of powders and salts. The protein expression level also showed a concurrence pattern where up to 48-hour incubation period, cav1 was still down-regulated regardless the metal source. Interestingly, cav3 showed the same expression pattern to that of cav1, suggesting a general response of caveolin protein family towards oxidative stress generated by the presence of metallic elements within the cell culture medium. This finding suggests the potential of caveolin protein family as a biomarker for biocompatibility test of BMs.

Chapter 6

Discussion

6.1 General Discussion

This doctoral project constitutes a key step within the developmental map of BMs for cardiovascular application that is presently being conducted in the Laboratory of Biomaterials and Bioengineering (LBB), Laval University. During BMs development, it is of importance to verify the biocompatibility of a chosen material even from the very beginning of the process. This would give precautions to the elemental choice of BMs avoiding future issue regarding the toxicity of BMs as implantable materials. The project will accommodate an efficient fabrication method, since it will provide hints at the potential use of in-development BMs for cardiovascular stent application. Previously, the LBB has developed pure electroformed iron [8] and iron-manganese alloys [127]. Nowadays, the LBB is intensively exploring the feasibility of several materials such as iron-phosphorus alloy, iron-cobalt alloy, and iron-manganese-carbon alloys for cardiovascular stent application. Development of those alloys will depend on biocompatibility assessment prior to be considered as potential candidates for stent as shown in Figure 6.1.

This doctoral project delineates the pertinence of conventional biocompatibility assessment methods for BMs. It emphasizes the importance of considering the continuous release of degradation products in this assessment. Cardiovascular stents are small in size, however the continuous release of degradation products may affect adjacent cells. The release of metallic ions may interfere the charge balance of the surrounding cells, which could lead to oxidative stress. The stress could be strong enough to damage cells. The stress could also be latent and would not give any observable morphological change to the cells although they are internally stressed. This could lead to chronic damages in the future, which most likely more detrimental. Therefore, conventional methods to assess the biocompatibility of BMs need to be

adjusted to evaluate the effects of continuous exposure of the degradation products on the surrounding cells. For this reason, gene expression profiling was proposed as a new approach to assess the biocompatibility of BMs. Through the gene expression study, latent internal stress can be detected since small imbalance in gene expression is measurable [10]. Therefore, it improves the conventional methods to be more sensitive in assessing the cellular state in presence of BMs.

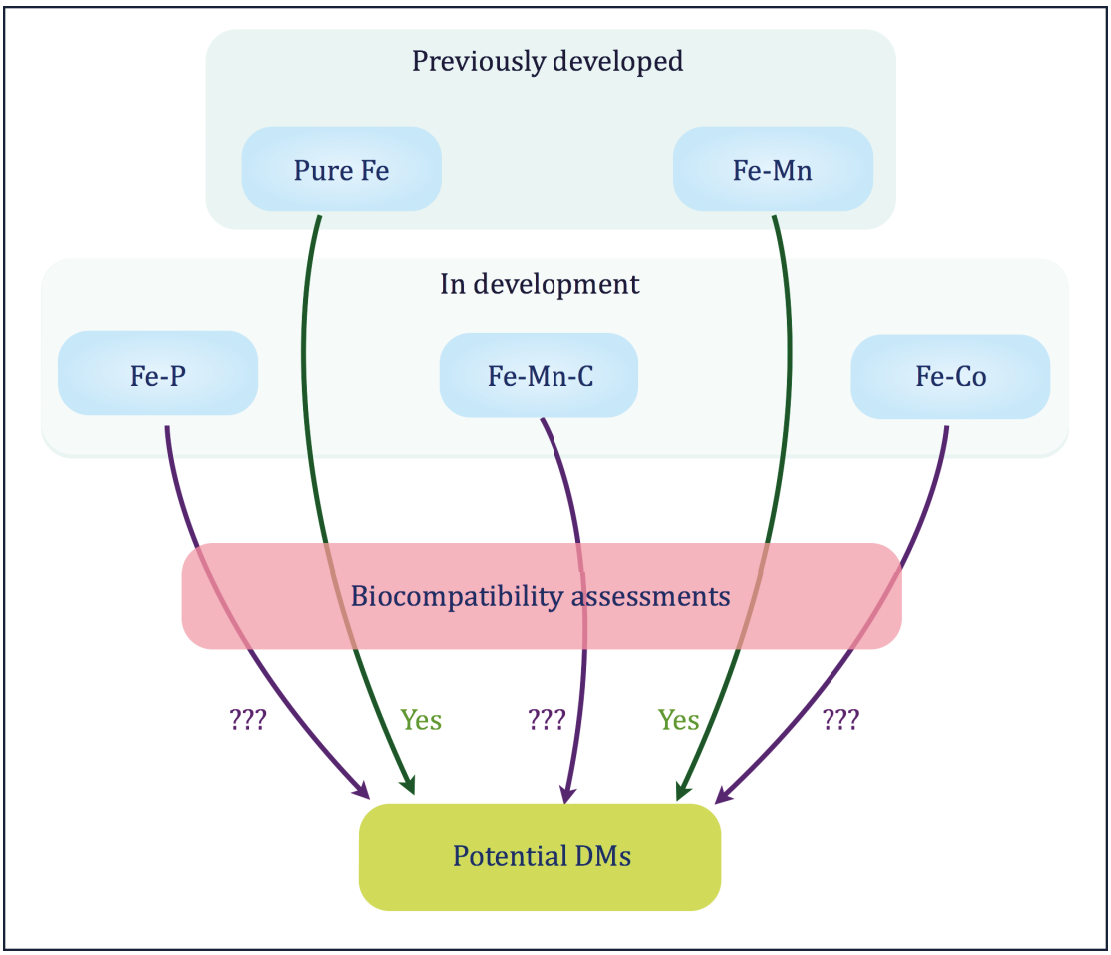

Figure 6.1. Exploration of BMs at LBB. Biocompatibility assessment constitutes an essential step in materials development confirming their potentials as BMs. Pure iron and iron-manganese alloys were previously developed at LBB have shown potential as new BMs. More materials such as iron-phosphorus, iron-manganese-carbon, and iron-cobalt are still in the developmental stage at LBB.

6.2 Host response and toxicity

Metals play important roles in human body including molecular transports, enzyme co-factors, as well as in DNA and RNA structures. Deficiency or absence of some metal elements could lead to biochemical changes or abnormalities. Those metals are categorized as essential elements, which include iron, zinc, copper, chromium, cobalt, molybdenum, and selenium. The main source of metal elements is through the food intake. Indeed, some of the elements such as iron and magnesium often do not meet the daily requirement, which then non-dietary supplemental source is needed. Being surpassed by the daily intake of iron and magnesium, about 40 mg and 400mg respectively, a single stent of about 40 mg pure iron or magnesium is considerably acceptable. However, the accompanying elements for iron and magnesium-based BMS such as manganese [9], cobalt [160], palladium [161], etc. might induce potential toxicity to the human body.

In assessing the biocompatibility of BMs, focus needs to be paid to the toxicity of the degradation products such as metallic ions and metal hydroxide. Even though the systemic toxicity seems to be unlikely, local toxicity maybe of importance. Subsequently, the ability of degradation products to interact with cellular homeostasis including metabolic activity and proliferation rate can be of importance. Moreover, inflammatory response such as macrophage activation in the presence of BMs and their degradation products are to be assessed.

6.3 Important parameters in assessing the biocompatibility of BMs

Generally, biocompatibility refers to the ability of a material to perform a particular function within living tissues accompanied by appropriate host responses. Classically, biocompatible materials were chemically inert. A material should not be releasing noxious agents or degrading biological molecules coming in contact with its surface thus causing tissue necrosis and persistent inflammation [162]. However, the definition of biocompatibility has evolved. Nowadays, biocompatible materials extent to bioactive materials, which present certain active chemical groups to the

surrounding tissue or undergo changes in their chemical composition and release certain agents to achieve a specific biological response [3].

Since biocompatibility involves a complex measurement, each assessment study should suggest its own working definition of biocompatibility. A single result from an assessment could not be used to generalize the overall performance of a material since the results may not be as expected. There are several factors that affect the biocompatibility in general, which include [162]:

1. Soluble agents released by the implant, e.g. ions or molecular fragment.
2. Insoluble particulate material released from the implant, e.g. wear debris.
3. Alterations in the strain distribution in tissue due to the mismatch in modulus elasticity between the implant and the surrounding tissue.

In the case of degradable metallic stents, the three mentioned factors can determine their biocompatibility. Considering that elemental choices used for degradable metallic stents such as iron and magnesium—are essential elements for human body and the materials are degraded progressively, small dimension of stents (40 mg), thus systemic toxicity is extremely unlikely [18]. The key point of BMS biocompatibility is closely related to their alloying elements. Alloying elements such as manganese [4] and cobalt [160] could shift the toxicity level of iron and magnesium. As previously reported, iron did not induced significant 3T3 cell counting up to 48-hour incubation period, while pure manganese alone had a significant effect to the same cell line. Putting the two elements as an alloy, it affected its toxicity [127].

6.4 *In vitro* system to assess BMs biocompatibility

The spectrum of *in vitro* test system ranges from cell death, alterations of cell adhesion, cellular proliferation, and biosynthetic activity. Cell death may be noticeable by direct observation under the phase-contrast microscope. Cell death is often signalled by cellular detachment from the substrate, nuclear shrinkage (pyknosis), and cytoplasmic fragmentation. In the case of cardiovascular stent, failure of blood cells attachment is of preference in order to avoid the occurrence of thrombosis [163]. Cell proliferation is often involved in biocompatibility test series. Decreasing cell count is often used as a trivial technique to conduct biocompatibility assessment. Alteration of biosynthetic activity is often measured as a biocompatibility parameter. The inhibition of synthetic function and cell death will classify the tested materials towards incompatibility, while minimal alteration might be acceptable and classified as biocompatible. Interestingly, a material could exert a stimulation of a certain desirable parameter and considered as bioactive. For example, a material might increase the adhesion of endothelial cells favouring a rapid endothelialisation after stent deployment or a material might increase the adhesion of osteoblasts to the bone implants favouring osteointegration [40].

In vitro systems have been applied for the biocompatibility assessment of various implants. In the case of degradable metallic stents, primary cultures of human SMCs and endothelial cells have been used to assess absorbable metallic stent (AMS, Lekton Magic) in comparison to gold standard material, stainless steel SS316L. It was found that magnesium-based alloy with yttrium and rare earth elements showed a strong inhibition of SMC proliferation (80%) and moderate reduction of viability (20%). Interestingly, no significant difference was observed for endothelial cells proliferation and viability in the presence of the alloy compared to that of SS316L [46]. These results have shown the potential of this alloy as a stent material. It was subsequently used for the fabrication of AMS. In another case an *in vitro* system using primary culture of human vascular SMCs allowed the assessment of ferrous ions acting as the degradation product of iron-based degradable metal for cardiovascular stent. It was shown that the presence of ferrous ions could reduce vascular SMCs proliferation rate

by influencing growth-related gene expression and therefore plays a potential role in avoiding restenosis as iron-based cardiovascular stent deployed [18].

6.5 Perspective on gene regulation-based analysis

Since the toxicity of degradable metallic stents and their degradation products are important parameters to be assessed to confirm their biocompatibility, and that the available conventional methods to assess stent materials are inappropriate to be applied for degradable metallic stents, there is a need for new approaches. Gene expression profiling study has been suggested to provide more comprehensive mechanism to the toxicity of a material, since it measures the cellular response prior to the phenotypic responses [162]. Hence, the use of a biomarker to assess the toxicity of degradable metallic stents would be a pertinent step.

Since caveolin has been shown as a potential biomarker to assess the toxicity of degradable metallic stents, it can be further utilized to confirm the preliminary biocompatibility of the developing degradable metallic stents. The constant down-regulation of caveolin on vascular cells emphasizes of its specificity for stent application even though it has been shown as a toxicity marker on non-vascular cell lines. However, this stepping stone discovery needs supplementary evidence from *In vivo* experiments in order to confirm its validity as a biomarker for degradable metallic stents toxicity. Moreover, another discovery of more biomarkers would empower the results of caveolin expression pattern towards the toxicity of degradable metallic stents.

6.6 New approach in assessing BMs

The emergence of a new class of material poses new challenges for the assessment of their biocompatibility. This doctoral project explored a new approach to assess BMs. Biocompatibility tests for BMs constitute a new direction with the main challenge related to the continuous release of metallic ions as the degradation products. For this, finding a new approach to assess BMs was a major hurdle at the beginning of the doctoral project. Here, we proposed to study the gene expression profile of cells exposed to degradation products from BMs. Our goal was to identify genes that could serve as the biomarkers of biocompatibility in the presence of BMs.

There are at least five gene families that have been identified to qualify the cellular state in the presence of continuous degradation products [6]. Summary of their role is shown in Figure 6.2.

1. Antioxidant family

   The released metallic ions can form complex with metal chelators such as adenosine diphosphate (ADP) and ethylenediaminetetraacetic acid (EDTA) which catalyze the formation of reactive oxygen species (ROS) through the Fenton reaction. ROS can be harmful to cells since they can undergo a reaction with the deoxyriboyl backbone of DNA causing DNA strand break. A natural cellular mechanism against ROS production is via superoxide dismutase (SOD1) activity. Therefore, measuring SOD1 expression could help evaluate cellular oxidative stress.

2. Matrix metalloproteinase

   Matrix metalloprotease (MMP) play an important role in degrading extracellular matrix facilitating macrophage mobility towards the production sites of inflammatory mediators such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α). This role of MMP could be used to estimate the activity and severity of body response in the presence of continuous degradation products of BMs.

3. Tumour suppressor

p53, a tumour suppressor gene, leads to the arrest of cell cycle at the G1-S check point in order to allow DNA repair. The arrested cell cycle can be transient or permanent. If severe DNA damage are present, p53 lead to apoptosis. Therefore, modulation of p53 could be a marker to assess the BMs biocompatibility.

4. Oncogenes

Oxidative stress may cause DNA damage leading to mutations causing the development of cancerous cells. The genes that promote the cellular over proliferation are called oncogenes. Amongst them, c-fos, c-myc, and c-jun are well-studied oncogene family. Modulation of their expression could be useful to assess BMs.

5. Cytokines

Cytokines are inflammatory mediators produced during injury or infection. Cytokines that promote inflammation are called proinflammatory cytokines, whereas some are called anti-inflammatory cytokines. IL-1 and TNF-α are the principal proinflammatory cytokines for acute phase inflammatory response. Increased proinflammatory cytokine expression may indicate the progression of the inflammation. Therefore, their modulation of expression could also be useful in assessing the biocompatibility of BMs.

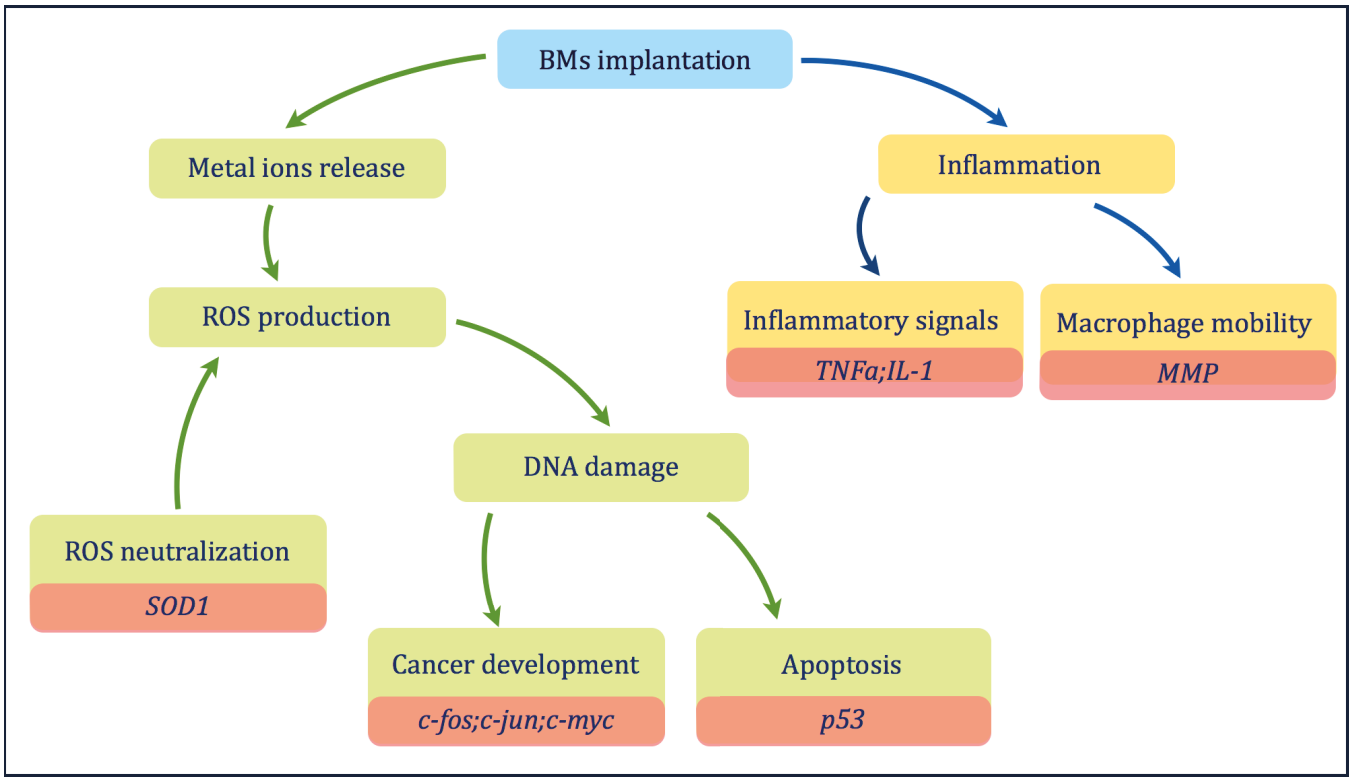

Figure 6.2. The role of potential genes to assess the biocompatibility of BMs. Following the implantation of BMs, metal ions will be released as degradation product. Metal ions could form complexes with metal chaelators and catalyze the formation of ROS. The expression of SOD1 will neutralize ROS production. Moreover, ROS production could lead to DNA damage, which subsequently induce apoptosis (marked by the expression of p53) and cancer development (marked by the expression of c-fos, c-myc, and c-jun). At the same time, BMs implantation could cause inflammation. This will activate the expression of inflammatory cytokines such as TNF-α and IL-1, and it will promote the mobility of macrophage, which is facilitated by the expression of MMP. Those marker genes are considered useful for assessing the biocompatibility of BMs.

6.7 Fibroblasts exposure to BMs

Following a literature research at the beginning of the project, an experiment involving fibroblasts exposure to BMs was conducted. Mouse fibroblast cell line was chosen for their workability. As for the BMs, pure iron and iron-manganese alloy were chosen in the form of powder in order to provide maximum surface and culture medium contact. Tissue culture inserts with 3μm pore size were used to expose 75μm BM powders. Prior to the gene expression study of fibroblasts in the presence of BMs, cytostatic dose for each BM was determined. The cytostatic dose is an important parameter for this project, since an effective dose that gives enough influence to the cells is critical. Excessive dose would kill all the cells in culture and inadequate dose would not influence the cells at all, hence gene expression study would not be feasible. It was then found that the cytostatic dose for iron-manganese alloy was 5 mg/ml and 0.25 mg/ml for pure manganese. As for pure iron, a large span of doses did not influence cell counts. A dose of 3.25 mg/ml was then chosen for pure iron that corresponds to 65% iron content in iron-manganese alloy.

Following the determination of cytostatic dose for each BM, fibroblasts were then exposed and gene expression study was subsequently conducted. As much as 68 up-regulated genes and 54 down-regulated genes were modulated in the same manner regardless the type of BM being exposed. They were then considered as metal responsive genes and their potential as markers for BMs biocompatibility was explored in the subsequent step of this doctoral project. The exposure of the BMs significantly regulates the expression of cholesterol-related metabolism. This suggests that the presence of BMs might create an oxidative stress promoting an increase of cholesterol metabolism, which is one of the key components that determine membrane fluidity [18, 162].

Furthermore, cell cycle-related genes were also significantly regulated in the presence of BMs. The expression of DNA-damage-inducible 45 alpha (Gadd45a) was one of the most significantly up-regulated genes. The expression of Gadd45a is induced by p53 expression that promotes apoptosis as previously predicted in the previous project

(Figure 6.2). The induction of Gadd45a alters the formation of cyclin dependent kinase and cyclin active complex, which are needed to promote cell cycle progression [6]. Its regulation was significantly elevated up to 48-hour exposure time.

Similar pattern to Gadd45a expression was observed for other genes including caveolin-1 (cav1), an integral membrane protein found in caveolae structure known as molecules transporter across endothelial barrier. Their expression was re-evaluated by quantitative real time – polymerase chain reaction (RT-PCR) technique and the results were in accordance with those from DNA microarray experiment. Moreover, cav1 became the point of interest within this doctoral project. Its expression was explored further up to protein level in order to confirm its expression consistency.

6.8 Caveolin as biocompatibility marker for BMs

Caveolin is the main structural component of caveolae membranes—little plasma membrane invaginations regulating cholesterol homeostasis, signal transduction, etc. Caveolin gene family consists of at least three genes expressed heterogeneously in different cell types. Caveolin-3 (cav3) is found specifically in muscle cells. While caveolin-1 (cav1) and caveolin-2 (cav2) are expressed in most cell types namely fibroblasts, adipocytes, pneumocytes, endothelials, etc. [142]. As previously discovered earlier in this doctoral project, cav1 was significantly regulated in the presence of BMs [164]. The gene expression study of fibroblasts exposed to BMs also shows a significant regulation of cholesterol-related metabolism [10]. Therefore, this finding suggests a relationship between BMs exposure, cav1, and cholesterol metabolism. Cav1 as the marker for caveolae structure, which supports cholesterol homeostasis, was clearly modulated in the presence of BMs. This fact strongly suggests the potential of cav1 as biocompatibility marker for BMs.

Subsequently, the project explored the consistency of cav1 modulation in presence of BMs. Different source of metal ions, $FeCl_2$ and $MnCl_2$, were used as comparisons to metal powders to confirm cav1 modulation pattern. Increase in gene expression of

cav1 also extended to protein level. The results showed that cav1 gene expression was down regulated in the presence of manganese powder and iron-manganese alloy up to 48 hours. Similar pattern was observed for cav1 protein level in the presence of manganese and iron-manganese powder. The evidence of cav1 potential as a biocompatibility marker for BMs was empowered by similar pattern showed by smooth muscle cells (SMCs) in the presence of BMs. Moreover, metal salts exposure gave a similar pattern to that of metal powders. Hence, the exposure of BMs decreased the expression of cav1 regardless the cell type and the source of metal ions [164]. Since caveolin is known as the principal structural protein of caveolae which has important roles for plasma transport and signalling, its down regulation pattern in the presence of BMs suggests a protection mechanism against soluble metal ions through deactivation of endocytic capacity of caveolae.

6.9 *In vivo* and clinical study for BMs biocompatibility

In vivo models give superior understanding of biological responses towards implant materials compared to *in vitro* models. They consist of complex systems of cellular interactions, hormones, dynamic blood circulation, excretions, etc., which are absent within *in vitro* models to possibly leading false negative results. It was reported that a variety of polymers in powdered form were mildly toxic to mouse fibroblast cells which their proliferation rate was decreased. On the other hand, *in vivo* study showed moderate toxicity when the same materials were implanted intramuscularly in rat model. Similarly, it has been reported that *in vitro* model of fibroblast and epithelial cells showed a higher number of materials which give growth inhibition compared to *in vivo* intramuscular rabbit model. It was then proposed that within *in vitro* model, the cells were not as dense as within an *in vivo* model. Thus the cells within *in vitro* model were particularly more vulnerable as the extent of cell-cell cooperation is at a minimum [165].

Experimental cost and ethical issues of animal testing often hold down the development of *in vivo* models. Animal of choice often adds a particular consideration,

as relevance to that of human system is important. Small animal models such as rodents and rabbits have the advantages of being convenient and cost effective. However, despite the essential structural similarity of the vascular walls, their cardiovascular system is not considered to be completely representative of the human situation. Thus, their biological responses within cardiovascular system will be different. Pigs appear to be a popular animal model to study the biocompatibility of cardiovascular stent materials due to its size and its capacity to implant in coronaries and atherosclerosis. Pure iron stents have been implanted in the coronary arteries, descending aorta, and iliac artery of the porcine model. It was shown that iron-based material did not induce any significant neointimal proliferation, thrombosis, local tissue necrosis, or pronounced inflammatory responses up to 12-month implantation period although a compelling degradation of the stent strut was observed. Similar results were found in New Zealand White Rabbit model up to 18-month implantation period, confirming the potentials of iron as cardiovascular stent material [77].

As seen in Figure 6.3, iron was the first metal being implanted in animal model. Iron stents were implanted in the descending aorta of New Zealand white rabbit for 18 months [77]. Subsequently, pigs became the most popular animal model since its cardiovascular system resembles to that of human. Moreover, coronary artery and descending aorta are the most popular implantation sites in the porcine model. In contrast to iron stent, magnesium stents have been implanted in human. The implantation sites included lower limb artery, pulmonary artery, aorta, and coronary artery. Similar to iron stent, porcine model is a popular model for magnesium stents implantation [21].

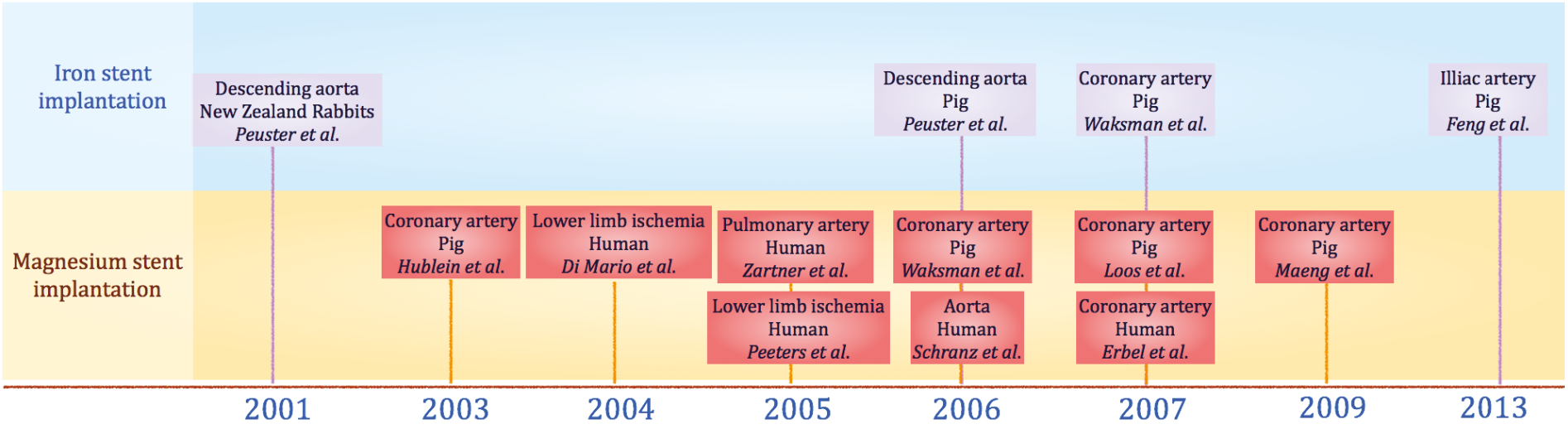

Figure 6.3 Implantation of iron and magnesium stent in human and animal model. Despite its comparable mechanical properties to that of stainless steel, no human implantation has been reported up to now. In contrast to iron stent, magnesium stent has been implanted in human lower limb artery, pulmonary artery, aorta, and coronary artery. Porcine is popular animal model since its cardiovascular system resembles to that of human [25].

6.10 Degradation mechanism of BMs

Biodegradation of BMs plays a key role in determining the biocompatibility. Through the corrosion process, BMs would dissolve themselves through time, leaving a fully remodelled artery. The dissolving process of BMs generates degradation products in the form of ions, hydroxides, gases, and particulates. Those degradation products could potentially harm the surrounding cells especially if they are generated abundantly and within a relatively short period. In order to understand how BMs could harm the surrounding cells, it is of importance to understand the mechanism behind their biodegradation. The mechanism of BMs biodegradation can be separated into several steps as followed [127]:

1. Initial corrosion reaction.

   Following the BMs deployment into the artery, it is oxidized to metallic ions in anodic spots according to equation (1). This anodic reaction generates electrons, which are subsequently engaged to cathodic reaction that reduces the oxygen that dissolved in the body liquid according to equation (2). Those reactions occur thorough the surface due to the different potential between the metal matrix and intermetallic phase (grain boundary).

   $$X \rightarrow X^{2+} + 2e^{-} \quad (1)$$

   $$2H_2O + O_2 + 4e^{-} \rightarrow 4OH^{-} \quad (2)$$

2. Formation of hydroxide layers

   Metal ions from equation (1) then react with the hydroxyl ions from equation (2), generating insoluble hydroxide following equation (3) on the surface.

   $$X^{2+} + nOH^{-} \rightarrow M(OH)_n \quad (3)$$

3. Formation of pits

   Since there is unequal formation of hydroxide layers on the surface, chloride ions can penetrate to compensate the increase of metal ions beneath the hydroxide layer forming metal chloride. It is then hydrolysed by water resulting free acid (equation 4), which leads to autocatalytic reaction and end up to the formation of pits.

   $$X^{2+} + 2Cl^{-} \rightarrow XCl_2 + H_2O \rightarrow X(OH)_2 + HCl \quad (4)$$

4. Formation of calcium/phosphorus layers

   Calcium and phosphate based apatite are then deposited on the undissolved hydroxide layers due to the saturation of calcium and phosphate in the body fluid and localized pH changing.

5. Degradation layer disintegration

   As the degradation layers are formed, surrounding cells move and embody the BMS. This might cause the disintegration of the degradation layers into irregular particles. The particles maybe enclosed by the fibrous tissue of macrophage dependently to their size. The BMs are further degrading progressively based on the previous steps until they are completely disappeared.

6.11 Limitation of the project

6.11.1 Cell culture system

The current understanding of biological process within this project was mainly based on the studies of homogenous population of cells cultured on flat surface using cell culture flask and multiwall plates (2D culture). The cell culture system was static requiring the change of culture medium frequently to provide the cells with fresh nutrients and also to remove metabolic waste. However, tissues and organs within the body are in 3D structures and they are continuously perfused by the blood circulation network. Moreover, it is generally recognized that there is a significant difference in cell behaviour and functions between flat layer of cells sitting in a medium bath and a complex three-dimensional tissue fed by blood circulation found in the body [166, 167]. Tissue specific architecture; mechanical; biochemical cues and cell-cell interaction are lost under the simplified 2D culture.

6.11.2 Fibroblast cell culture

Arteries constitute of three different layers containing specific cell type for each layer. The most inner layer called tunica intima consisting endothelial cells that lines the arterial lumen. The second layer is called tunica media consisting smooth muscle cells which are responsible for the contractibility property of the artery. The most outer layer is called tunica externa consisting fibroblast cell population. When a stent is being deployed within the artery, it will tear up the endothelial layer exposing itself to be in contact with endothelial cells and smooth muscle cells. It is unlikely that the stent will touch fibroblast cells population within the most outer part of the artery structure. However, fibroblast cells will play important roles in the process of arterial remodelling following the stent deployment. They are mechanoresponsive cells allowing the conversion of mechanical signal from stent deployment into biological events such as the production of growth factors, cytokines, and extracellular matrix components that are essential for cellular migration [42].

6.11.3 DNA microarray experiment

DNA microarrays is a powerful method for the global analysis of gene or protein content and expression, opening up new horizons in molecular and physiological systems. Such widespread adoption of DNA microarray technology in both industry and many academic research laboratories is largely due to its capacity to provide researchers the opportunity to quickly and accurately perform simultaneous analysis of literally thousands of genes in a parallel manner or even entire genome of an organism (e.g. bacteria, yeast, virus, protozoa, mouse or human) in a single experiment, hence providing extensive and valuable information on gene interaction and function [168]. Despite their wide spread use, DNA microarray have a few disadvantages such as being expensive and sequence homologies between clones representing different closely related members of the same gene family may result in a failure to specifically detect individual genes and instead may hybridize to spot designed to detect transcript from a different gene. This phenomenon is known as cross hybridization [169]. The high-throughput nature of this technology combined with the expected abundant of data results (tens of thousand mouse genes were involved in the project) in a high opportunity for errors. To ensure the accuracy and reliability of the resulting data, it is essential, therefore, that experiments are tightly regulated and quality controlled. At present, the sophistication of microarrays renders this a costly technology and consequently it is only available to specialized institutions. In this project, the experiment was conducted at Genome Quebec, Montreal. Microarray technologies, however, are rapidly improving and the costs of the technique continue to fall, thus paving the way for wider access and more-generalized usage.

6.11.4 Bioinformatic programs

The amount of data produced by microarray experiments is daunting even to statisticians. Therefore handling numerous inputs to be analyzed using certain bioinformatics software is very challenging with significant occurrence of oversight. Several bioinformatics programs were involved in this project in order to cross check significant results and minimizing the errors and miscalculations [170]. An important pre-processing step, often termed as "low-level" analysis, involves the so called "normalization", which removes systematic biases due to imperfect experimental conditions, and quality filtering, which picks out "bad spots" and removes artifacts. For example, due to hybridization bias and other reasons the mRNA levels labeled by Cy5 may be systematically higher than that labeled by Cy3. The first normalization method is to subtract a constant from the expression measurements of all the genes. But this approach can be problematic due to certain expression intensity dependent biases. More sophisticated statistical approaches using "rank invariant" genes or robust curve estimation are often more appropriate. A central task intended for the microarray experiment is to find genes that are differentially expressed in the two samples (or types of cells) [171]. Suppose that the identical microarray experiment is repeated p times (e.g. leukemia cells from p patients compared with p wild types). Then, we obtain a dataset (m_{ij}; i=1,…, G, j=1,…,p), in which m_{ij} is the expression ratio of gene i in j^{th} experiment. The number G ranges from thousands to tens of thousands, while the number of replications p can be as low as a few. The statement "differentially expressed (DF)" simply means that, mathematically, E (m_{ij}) 0. The standard t-test is an obvious first attempt for recognizing DF genes and has been implemented in all commercial microarray analysis packages. But the distributional assumption and the problem of multiple testing make the statisticians wonder how reliable the t-tests are in and what the "false discovery rate" is. Recently, empirical Bayes and parametric Bayes methods have been suggested to tackle these questions [172].

Conclusion

Biocompatibility refers to the ability of a material to perform a particular function within living tissues with appropriate host responses—minimum inflammatory and toxicity reactions both locally and systematically. Biocompatibility is a requirement for todays implants including cardiovascular stent materials. Guidelines for biocompatibility assessment have been issued by the International Standard Organization (ISO), however they are not specifically intended for BMs. Standard methods to assess BMs biocompatibility are needed in order to assess their performance and interaction with the biological system that determine their biocompatibility. These will help to identify the candidate materials to be considered as biologically compatible.

Biocompatibility tests of BMs should envisage the continuous release of metallic ions as degradation products. The release of metal ions induces the formation of reactive oxygen species (ROS) through the Fenton reaction. The presence of ROS is closely related to DNA damage leading to cell death or cellular over proliferation. Therefore, genetic regulation plays important roles in dealing with the degradation products.

Adverse effect of degradation products could be strong enough to damage the cells, an easy-to-observe phenotype. This is what the conventional methods are likely able to do. The adverse effect of the degradation products can be invisible morphologically even though cell physiology is disturbed. This disturbance may remain undetected until it eventually lead to chronic damage. Conventional methods to assess the biocompatibility of BMs thus need to be adjusted in a way that continuous release of degradation products is taken into the account. Here we proposed gene expression profiling as a new way to assess the biocompatibility of BMs. This can be performed by assessing the cellular response towards degradation products. Altered cellular behaviour resulting from the gene expression regulation can be used as a new approach to investigate the biocompatibility of BMs. Therefore, it complements the conventional methods with a more sensitive detection limit.

1. New approach to assess BMs biocompatibility

The change of cellular behaviour resulting from gene expression regulation is a potential approach to assess the biocompatibility of BMs. With this approach, up- or down-regulated genes related to the cellular responses due to the presence of degradation products could eventually be used as biomarkers. This approach investigates the biocompatibility at the first level of detection since it measures mRNA levels of genes responsible for cellular behaviour. Thus, this approach could give new information of BMs biocompatibility, where previous conventional *in vitro* test methods remained silent. Specific gene families mentioned to be related to tissue remodelling, cell cycle control, inflammatory signals, and ROS-neutralizing activity are considered to support the biocompatibility facts since genetic regulations are the basic mechanism of the cellular function in response to the presence of foreign bodies. This approach is expected to give advantage for the design and development of metallic materials specific to BMs for stent application.

2. Fibroblasts exposure to BMs

Pure iron did not significantly influence the gene expression profile of fibroblasts. On the other hand, manganese showed a marked cytotoxicity at relatively low concentrations. Some iron homeostasis genes were regulated when exposed to the alloy, although their modulation in expression was not significantly different. It was notably observed that the exposure to the alloy regulates cholesterol metabolism since cholesterol is one of the key components that determines membrane fluidity. Exposing cells to a degradable alloy could create membrane instability due to the oxidative stress induced by the released metallic ions. Since the effect made by iron-manganese alloy was also observed in the manganese exposure, it suggests that manganese, as a component of the alloy, was responsible for this result. At the gene expression level, GADD45u, DDIT3, TFRC3, TCF3, CAV1, MAGED2, ACAT2, and CXCL12 showed a stable expression pattern up to 48 hours, suggesting the need for further study to confirm their potential as biomarkers for BMs.

3. Caveolin as a marker for BMs biocompatibility

Gene expression study showed that cav1 was constantly down regulated in the presence of metallic elements both in the form of powders and salts. The protein level also showed a similar pattern where up to 48-hour incubation period, cav1 was still down regulated regardless the metal source. Interestingly, cav-3 showed the same expression pattern in SMCs to that of cav1 in fibroblasts, suggesting a general response of caveolin protein family towards the oxidative stress generated by the presence of metallic ions within the cell culture medium. This finding suggests the potential of caveolin protein family as a biomarker for the biocompatibility test of BMs.

Therefore, gene expression study has been successfully shown to be potential as the new approach to assess the biocompatibility of BMs. Caveolin gene family, specifically Cav1, has been identified as an appropriate biomarker for BMs. The results were proven by the experiments involving fibroblasts and smooth muscle cells with different sources of metal ions and different incubation periods.

Future works

1. Complementary markers to cav1

Although the expression pattern of caveolin was shown to be constant—a promising result for the toxicity marker of BMs, it would be empowering to have other markers to complete the assessment system leading to more accurate results in determining the toxicity of BMs. To do so, the suggested potential biomarkers generated from gene expression study such as GADD45α, DDIT3, TFRC3, TCF3, MAGED2, ACAT2, and CXCL12 need to be explored. Additionally, there are more gene family to explore as complements to caveolin gene. There are at least five gene families have been identified to determine the cellular state and behaviours in the presence of continues degradation products: antioxidant genes, matrix metalloproteinase genes, tumor suppressor genes, oncogenes, and cytokine genes.

2. Co-culture system to assess BMs

In the living tissue, one cell type is interacting with other cell types. Therefore, it is important to investigate the behaviour of the cells when they are interacting with other type of cells as opposed to study their behaviour as a single culture. There are at three cell types can be found within the arterial structure: endothelial cells, smooth muscle cells, and fibroblast cells. Each cell type has different characteristic in terms of physical and biochemical properties. However, they are interacting with each other through a complex network involving the production of various growth factors, cytokines, and extracellular matrix. Endothelial cells are actively producing NO (nitric oxide) which plays a key role in avoiding the adhesion and activation of platelet on the vessel surface. At the same time, NO plays a regulatory function since it promotes the quiescence stage of SMCs (avoiding the proliferation of SMCs) [173]. Similarly to ECs, fibroblast cells also interact with SMCs. Fibroblast cells are known as mechanoresponsive cells. They allow the mechanical signal that may come from the stent deployment into biological events such as the production of extracellular matrix which is essential for the migration of SMCs [42].

3. 3D cell culture to assess BMs

Most of the cells require cues from 3D environment to form relevant physiological tissue structures *in vitro*. Increasing studies indicated that 2D cell cultures cannot reflect the physiological complexity of real tissue and their use in cell-based assays might, to some extent; result in errors in predicting tissue-specific responses [166, 174]. Cells cultured in 2D formats largely undergo proliferation and de-differentiation and consequently loss their functions. In contrast, cells cultured in 3D matrix showed significant difference in terms of proliferation, differentiation, morphology and functions. Cells within a tissue interact with neighboring cells and with the ECM through biochemical and mechanical cues. Cell–cell and cell– ECM interactions establish a 3D communication network that maintains the specificity and homeostasis of the tissue. The tissue architecture and geometric property, and mechanical stress and fluid flow direct cell morphogenesis and functions. The limitation of conventional 2D culture due to 'over-simplification' has long been recognized. The availability of standardized consumables, instruments and equipment make it difficult to move away from such a well-established methodology. Various improvements and further developments to capture more physiological cell functions using the 3D culture are interesting challenges to be explored

4. Involvement of bioreactors to assess BMs

Continuous supply of fresh culture medium is needed in order to remove the metabolic waste in addition to deliver the nutrients for the cells (perfusion effect). Accumulated metabolic waste in the culture medium affect the cellular behaviors creating suboptimum culture condition. Bioreactors are best applied with 3D culture system mimicking *in vivo* situation up to some extent. The combination of 3D culture with perfusion is powerful and can potentially lead to the creation of a variety of *in vitro* tissue models for different applications [175]. Bioreactors are defined as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature,

pressure, nutrient supply and waste removal) to generate microenvironment [175, 176]. *In vitro* 3D cell culture within bioreactor systems that can mimic organ and tissue structure and function *in vivo*, will be of great benefit for drug discovery and toxicity testing. Development of *in vitro* 3D human tissue/organ models is of particular important as rodents or other animals can metabolize and respond to drugs or toxic materials differently from humans. The 3D cell culture within bioreactor systems could potentially replace some animal testing and provide more reliable and predictive data for the early screening of human drug and materials toxicity in the preclinical. The earlier that *in vitro* toxicity testing is performed the more it can save in terms of time and cost associated mirroring *in vivo* state. Perfusion is also important for toxicity testing since the dosage and the dosing protocols that can be conveniently manipulated externally without disturbing the cultured cells. Moreover the concentration profiles around the cultured cells are well defined and known. Hence a quantitative link between the degradation products and cellular responses can be established.

5. Proteomic study to assess BMs

Proteomic refers to the analysis of genomic complements of proteins. Proteomic has emerged into the scientific scene with significant development over the past few years. But whereas every fragment of DNA behaves biochemically much like any other, proteins possess unique properties creating an enormous hurdle for methodologies that seek to assign an activity to sets of proteins that may number in the thousands. The analysis of protein expression profiles provides additional information to genomic and mRNA analysis, since (1) a proteome is dynamic and it is spatially and temporarily expressed (e.g. proteins in the nucleus for transcription and mitochondrion for energy regeneration); (2) proteins operate in 'clusters' or 'modules' made of various species of interacting molecules that carry out cellular functions, such as signal transduction; and (3) protein carry dynamic (e.g. phosphorylation) and static modification (e.g. disulfide linkage) that may not be apparent from genomic information or from mRNA abundance [177]. Therefore, it is an interesting challenge to conduct proteomic study in accordance to the established

genetic regulation study within this project.

6. *In vivo* study

One of the challenging future works constitute *in vivo* experiments confirming the expression pattern of the identified gene markers such as CAV1, GADD45α, DDIT3, TFRC3, TCF3, MAGED2, ACAT2, and CXCL1. Additionally, there are more gene family to explore as complements to those genes including antioxidant genes, matrix metalloproteinase genes, tumor suppressor genes, oncogenes, and cytokine genes. Their expression pattern within the animal models can be assessed through immunostaining following an implantation study. However, an appropriate animal model needs to be first established. Interesting small animal models for preliminary *in vivo* study include rats and rabbits models, while bigger animal models that represent well the cardiovascular system of human include pigs and dogs models.

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Online resources:

1. http://www.nlm.nih.gov/medlineplus/ency/18020.htm; March 2014)

2. http://www.nlm.nih.gov/medlineplus/atherosclerosis.html; March 2014)