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BIOFUNCTIONALITY AND IMMUNOCOMPATIBILITY OF STARCH-BASED BIOMATERIALS

Submetida por

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Para candidatura à obtenção do grau de Doutor em Ciência e Tecnologia de Materiais, área de conhecimento em Biomateriais

Julho 2004

To my Parents

(Aos meus Pais)

“Failure is the preamble to success. Most first efforts don’t work. If you persist, you’ll eventually figure it out”

-Thomas Fogarty

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ACKNOWLEDGEMENTS

To thank everybody that supported and accompanied me during the work described in this thesis and its preparation is probably the most difficult part for me. Nonetheless, not wanting to disregard anyone, I have to reveal some names.

I would like to address a very special recognition to Dr. Rui Reis, my supervisor, without whom I would not had the chance to concretise my dream of “doing real research”. Thanks to Rui I had great opportunities. During all this years working with him I was always learning and growing up in Science, feeling that with his support and advise I could overcome challenge after challenge. The most recent battle was the preparation of this thesis during which he knew when and how to motivate me to proceed and make another conquest.

I also have to express some words of recognition to Dr. John Hunt, my co-supervisor at the University of Liverpool. He accepted to co-supervise this work without knowing me. Throughout my stays in Liverpool he was always extremely supportive and helpful, trying to make me fell at home. He was always able to rearrange his timetable to go to the lab or to discuss and give suggestions about the work. Working in Liverpool was a great opportunity and I enjoyed it very much.

While I stayed in Liverpool I made many friends. They witnessed and shared my joys and despairs during the long days of lab work. In particular I am very grateful to Jude, Paul, Debbie, Lisa and Jill. A special word to Chris Oliver, who taught me how to work in a cell culture lab and gave me valuable advices in the beginning of this work, to Sandra for her help with the scanning electron microscopy and to Shirley for her day to day help. Finally I would like to acknowledge everybody from the Clinical Engineering Department; to several persons, thank you for the experimental help and to all of them for the support.

I also have some friends in the 3B’s research group to whom I would like to acknowledge. Manuela is responsible for my awareness of the “Biomaterial’s World”. She has been my confident and adviser, helping me to take the best decisions and to achieve my objectives. Catarina, who worked with me in Liverpool, helped me to forget the most difficult phase of my PhD and to move forward. She also gave me some experimental help for which I am very grateful. Belinha and Helena have been the discrete but present friends who congratulated in the best and gave support in the worst situations. I would also like to thank all my other colleagues from the 3B’s research group for their concern and understanding. I would also like to thank Tírcia for her help and understanding in the final stage of preparation of this thesis.

I must acknowledge the Department of Biology of the University of Minho, in particular to Dra. Olga Coutinho, for facilitating the access to the animal cell culture lab to perform some of the work described in this thesis.

I would gratefully acknowledge the Portuguese Foundation for Science and Technology and the Portuguese Programme PRAXIS XXI for awarding me a PhD Grant.

I cannot forget my parents. During the last four years they were unconditionally present supporting me and giving me strength to pursue my dreams. Although we were not always in agreement, this PhD is also their dream therefore I dedicate it to them.

To conclude, I have to express my gratitude to my husband Helder. Without him and his support throughout the many and long months apart I would never be strong enough to overcome the worst moments and to work the way I did. Without his persistence, comprehension and encouragement this dissertation would still be a plan.

SHORT *CURRICULUM VITAE* OF ALEXANDRA P. MARQUES

Alexandra P. Marques was born on 1975 in Águeda, Portugal. At the present she lives in Porto and works in Braga, as a researcher, in the 3B's Research Group (Biomaterials, Biodegradables & Biomimetics).

Alexandra P. Marques background includes: i) a four years graduation in Biochemistry, Faculty of Sciences, University of Porto (with 14 over 20); ii) a one year specialisation course as part of the Biomedical Engineering Master/Doctoral Programme, Faculty of Engineering, University of Porto (with 15 over 20). She has just submitted her PhD thesis on Materials Science and Technology – Biomaterials to University of Minho, Portugal, which was prepared in cooperation with the University of Liverpool, United Kingdom.

During the last year of her graduation, Alexandra P. Marques was involved in a research project in the veterinary field using her biochemical knowledge. Since 1998 she has been working with biomaterials, focusing the *in vitro* evaluation of the biocompatibility of natural origin polymers. She has also worked several periods abroad in the University of Liverpool in UK where she centred her research interests in the evaluation of the immunological response to biomaterials.

While researcher of the 3B's Research Group, she has been involved, at academic level, in the co-orientation of final year projects of under-graduated students as well as supporting practical lectures of Biomaterials of the Applied Biology course. She was also involved in the organisation of two NATO/ASI Advanced Courses, one in Tissue Engineering (2001) and the second one in Biomineralisation (2003) in Alvor, Portugal. Alexandra P. Marques was also involved in the preparation of the proposal of the most recent European Network of Excellence approved (EXPERTISSUES), coordinated by Rui L. Reis, the director of the 3B's Research Group. She was further implicated in the preparation of other research projects proposals both at European (framework VI) and National (Portuguese Foundation for Science and Technology) level.

As a result of her research work she has attended the most important international meetings in the biomaterials field both with posters and oral communications. She has published in refereed journals (4 papers, 4 submitted), as book chapters (4 *in press*) and in international conference proceedings (15 communications). A. P. Marques won the student prize at Tate- The Art of Tissue Engineering Seminar and Workshop in The Netherlands (2000) and was awarded an Honorary Mention by The Society for Biomaterials on the 29th Annual Meeting of The Society for Biomaterials in USA (2003).

LIST OF PUBLICATIONS

The work performed during this PhD resulted in the following publications:

PAPERS IN REFEREED JOURNALS

A. P. Marques, R. L. Reis, J. A. Hunt, *In vitro* evaluation of the biocompatibility of novel biodegradable starch-based polymeric and composite materials, *Biomaterials*, **23**, (2002), 1471-1478

A. P. Marques, H. R. Cruz, O. P. Coutinho, R.L. Reis, Effects of starch-based polymers and composites on the in vitro proliferation and viability of osteoblast-like cells, *Journal of Material Science: Materials in Medicine*, *submitted*

A. P. Marques, R.L. Reis, Hydroxyapatite reinforcement of different starch-based polymers affects osteoblast-like cells adhesion/spreading and proliferation, *Materials Science and Engineering C*, *in press*

A. P. Marques, R. L. Reis, J. A. Hunt, Evaluation of the potential of starch-based biodegradable polymers in the activation of human inflammatory cells, *Journal of Material Science: Materials in Medicine*, **14** (2003) 1-7

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BOOK CHAPTERS

A. P. Marques, J. A. Hunt, R. L. Reis, Natural origin degradable materials: the barrier or the passage through the immune system?, in: Biodegradable Systems for Tissue Engineering and Regenerative Medicine, Ed: R. L. Reis and J. San Roman, CRC Press, Boca Raton, USA (2004), 355-375.

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A. P. Marques, R. L. Reis, J. A. Hunt, Effects of starch-based polymers and composites on human leukocytes, 16th European Conference of the European Society for Biomaterials, London, UK, Sept., (2001), T8

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A. P. Marques, R. L. Reis, J. A. Hunt, Parallelism in the *In Vitro* and *In Vivo* Evaluation of the Host Response to Starch-Based Biomaterials for Regenerative Medicine, Regenerative Medicine: The Advent of Combination Products, Philadelphia, USA, October, (2004), *Submitted*

RESUMO

A procura de novos biomateriais que desempenhem funções específicas sem, no entanto, desencadearem respostas negativas nos hospedeiros constitui um desafio permanente e actual nesta área. Biomateriais degradáveis foram uma das soluções propostas e actualmente em aplicação mas, embora possuam vantagens inegáveis, também apresentam alguns problemas nomeadamente no que diz respeito aos seus produtos de degradação e respectivos efeitos negativos consequentes. Outros biomateriais, entre os quais polímeros de origem natural, foram propostos considerando que os seus produtos de degradação poderão ser incorporados nas vias metabólicas normais evitando efeitos secundários no hospedeiro.

Até ao momento, e apesar de todos os esforços e do grande número de dispositivos biomédicos desenvolvidos, o biomaterial ideal para uma aplicação específica ainda não foi encontrado. Estudos com polímeros biodegradáveis à base de amido demonstraram que estes materiais possuem propriedades promissoras abrindo novas perspectivas para a sua possível aplicação numa variedade de aplicações biomédicas. Assim, de modo a demonstrar que estes materiais têm de facto potencial para serem utilizados em, por exemplo, substituição óssea, sistemas de libertação controlada, cimentos ósseos e engenharia de tecidos, seria imperativo avaliar com maior profundidade a resposta biológica desencadeada pelos mesmos. Para tal foi delineado um plano de trabalhos com três objectivos principais: i) avaliar a citocompatibilidade dos polímeros e compósitos à base de amido com monitorização da citotoxicidade e análise da adesão e proliferação celulares nas suas superfícies. Foi dada particular atenção a osteoblastos considerando uma possível aplicação ortopédica para estes materiais; ii) estabelecer modelos *in vitro* para analisar e prever, tanto quanto possível, uma situação real de resposta inflamatória; iii) validar os resultados *in vitro* com um modelo *in vivo* já estabelecido em outros trabalhos de análise da resposta inflamatória a biomateriais.

Foram estudadas misturas de amido de milho com três componentes sintéticos (álcool etileno vinílico, acetato de celulose e policaprolactona) assim como os respectivos compósitos com percentagens crescentes de um cerâmico bioactivo (hidroxiapatite). Numa primeira fase de avaliação do efeito dos produtos de degradação dos materiais em estudo foi concluído que duas das misturas apresentam baixa toxicidade, mesmo inferior à determinada para o biomaterial biodegradável padrão Poly-L-Lactic Acid (PLLA). No entanto, os testes de adesão celular revelaram que, para além dos produtos de degradação, as propriedades das superfícies podem determinar se um material possui ou não as características apropriadas para a melhor resposta celular. O efeito da incorporação de HA nos materiais à base de amido foi também analisado concluindo-se que a presença deste

cerâmico não induz, de um modo geral, um efeito significativo no desempenho celular de células do tipo osteoblastos.

A avaliação *in vitro* da imunocompatibilidade dos polímeros e compósitos à base de amido foi efectuada numa segunda fase. Esta centrou-se essencialmente na identificação de variações na produção de radicais livres e enzimas degradativas por neutrófilos assim como na capacidade desses materiais activarem, *in vitro*, outras células do sistema imune. Na presença de alguns materiais, os neutrófilos foram estimulados a níveis muito inferiores ao seu potencial máximo enquanto que na presença de outros materiais as espécies reactivas de oxigénio produzidas pelos neutrófilos activados e que provocam lesões tecidulares, foram inactivadas por “scavangers” presentes nos materiais à base de amido. Verificou-se ainda que algumas citoquinas específicas não foram produzidas na presença de alguns dos materiais estudados enquanto que outras não foram mesmo segregadas em nenhuma das condições. A incorporação de hidroxiapatite nos polímeros à base de amido não afectou significativamente a activação das células do sistema imune ou resultou, dependendo da mistura em causa, numa menor activação.

De um modo geral, a avaliação da imunocompatibilidade *in vitro* permitiu concluir que os polímeros e compósitos à base de amido têm um fraco potential para desencader uma resposta inflamatória. Após implantação *in vivo*, estes resultados puderam ser confirmados validando os modelos experimentais estabelecidos *in vitro*. Macroscopicamente não foram observados sinais de uma reacção inflamatória considerável. O facto de não se ter formado qualquer exsudado celular, de os materiais terem sido encapsulados por uma cápsula fibrosa fina e da amplitude da resposta nos tecidos não ter sido muito forte para a maioria dos materiais em estudo, permitiu afirmar que os polímeros e compósitos à base de amido não induzem uma resposta inflamatória severa para os tempos de implantação estudados.

Em resumo, os estudos de citocompatibilidade e imunocompatibilidade demonstraram que os polímeros e compósitos à base de milho são biomateriais promissores. Em comparação com os biomateriais degradáveis actualmente em uso, possuem propriedades capazes de induzir um comportamento semelhante, ou mesmo melhor, em termos de citotoxicidade. Estes dados foram reconfirmados com a adesão e proliferação de células do tipo osteoblastos na superfície de alguns dos materiais à base de amido, que demonstraram ser comparáveis às observadas no PLLA, evidenciando a possibilidade de usar esses materiais em aplicações ortopédicas. As conclusões retiradas dos estudos *in vitro* e *in vivo* de imunocompatibilidade reforçam as observações das experiências de citocompatibilidade e em conjunto, evidenciam a possibilidade de utilização dos biomateriais à base de amido, com fraca capacidade de desencadear uma reacção inflamatória, em aplicações biomédicas.

ABSTRACT

There is an increasing demand for the development of new biomaterials that perform their function without eliciting negative effects in the host. Biodegradable biomaterials have arisen as the solution for some problems which currently involve traditional devices. However, and not neglecting the advantages of the degradable systems, some drawbacks have been found especially in terms of deleterious effects originated from their degradation products. Therefore, natural origin polymers are emerging within the biomaterials field. The rationale is that the incorporation of their degradation products into normal metabolic pathways will avoid secondary effects in the host.

So far, and although there are many biodegradable biomedical devices being used, the ideal biomaterial for a specific application is yet to be discovered. Studies with biodegradable starch-based polymers have demonstrated that these biomaterials possess a range of properties which make them suitable for different biomedical applications. Thus, to gain further inside in the suitability of these natural origin biomaterials for different biomedical applications such as bone replacement, drug delivery systems, bone cements and tissue engineering scaffolding, we considered that it was imperative to further assess the biological response provoked by them. For that the PhD work plan that culminated in this thesis was delineated having in mind three main goals: i) to assess the cytocompatibility of the starch-based polymers and composites by means of screening the cytotoxicity and evaluating cell adhesion and proliferation behaviour on their surfaces, particularly osteoblasts since we pursue a potential orthopaedic application for these materials; ii) to establish *in vitro* models to analyse and to try to predict, as much as possible, the real *in vivo* situation in terms of host response, thus to evaluate immunocompatibility of the materials; iii) to validate the *in vitro* results with an *in vivo* model previously used to study host response.

Blends of corn starch with three different synthetic components (ethylene vinyl alcohol, cellulose acetate and polycaprolactone) and their respective hydroxyapatite (HA) reinforced composites with increasing percentages of HA ceramic were studied. The short-term effects of the degradation products was analysed and it was possible to observe that two of the studied blends possess a low degree of toxicity, being even better than the gold standard biodegradable biomaterial. Nonetheless, cell adhesion tests revealed that surface properties can determine if a material has the appropriated characteristics for the best cellular behaviour. Generally, the reinforcement of the polymers with hydroxyapatite did not induce a significant effect in terms of improvement of osteoblast-like cells performance with the studied conditions.

The *in vitro* evaluation of the immunocompatibility of starch-based polymers and composites was focused on changes in the free radical and degranulation activity of neutrophils as well as on their potential to activate immune system cells *in vitro*. Neutrophils were in some cases

not stimulated to their potential maximum and in other situations scavengers of the reactive oxygen species, which are known to provoke tissue damage, produced by activated neutrophils reduced their negative action. In addition, specific pro-inflammatory cytokines were not produced in the presence of some of the studied materials while others were even undetectable under any of the stipulated conditions. In terms of inflammatory reaction, the presence of hydroxyapatite either did not induce a significant effect or resulted in low cell activation.

Thus, the *in vitro* results allow for concluding that starch-based polymers and composites possess a weak potential to break out an inflammatory response. After *in vivo* implantation no macroscopic signs of a considerable inflammatory reaction in any of the animals were observed, no cellular exudate was formed and a thin fibrous capsule surrounded all implants. Although there were some materials that stimulated stronger tissue responses, generally biodegradable starch-based biomaterials did not induce a severe reaction for the studied implantation times. The *in vitro* results were therefore confirmed by these *in vivo* observations which validate the established *in vitro* models.

In general, the cytocompatibility and immunocompatibility studies showed that starch-based polymers and composites are promising biomaterials. Comparatively to the currently used biodegradables, they possess properties that induce similar to better cytotoxicity behaviour. The adhesion and proliferation of osteoblast-like cells on some of these materials was also comparable to that of PLLA which demonstrates their potential to be used in orthopaedic applications. The *in vitro and in vivo* immunocompatibility remarks further support the suitability of starch-based biomaterials to be used in biomedical applications due to their weak potential to break out an inflammatory reaction.

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LIST OF ABBREVIATIONS

A

AaDSC - Acyl azide-crosslinked Dermal Sheep Collagen
AO - Acridine Orange
APC - Antigen Presenting Cells

B

BCA - Bicinchoninic Acid
BMP - Bone Morphogenic Proteins
BrdU - Bromodeoxyuridine

C

CAM - Cell Adhesion Molecule
CD - Cluster Designation
CPD - Critical Point Drying
CSF - Colony Stimulating Factor

D

DC - Dendritic Cell
DSC - Dermal Sheep Collagen

E

EB - Ethidium Bromide
EGF - Epidermal Growth Factor
ELAM - Endothelial Leukocyte Adhesion Molecule
ELISA - Enzyme Linked Immunosorbent Assay
EtO - Ethylene Oxide

F

FBGC - Foreign Body Giant Cell
FBS - Fetal Bovine Serum
FDA - Fluorescein Diacetate
FGF - Fibroblast Growth Factor
fMLP - Formyl-Methionyl-Leucyl-Phenylalanine
Fn - Fibronectin

G

GAG - Glycosaminoglycan
GDSC - Glutaraldehyde-crosslinked Dermal Sheep Collagen
GF - Growth Factor
G-CSF - Granulocyte Colony Stimulating Factor
GM-CSF - Macrophage Colony Stimulating Factor

H

HA - Hydroxyapatite
HDSC - Hiisothiocyanate-crosslinked Dermal Sheep Collagen

I

ICAM - Intracellular Adhesion Molecule
Ig - Immunoglobulin
IFN - Interferon
IL - Interleukin
IPN - Inter-Penetrating Network

ISO - International Standard Organisation

L

LC - Langerhan Cells
LDH - Lactate Dehydrogenase Activity
LFA - Lymphocyte Function-related Antigen
LIF - Leukaemia Inhibitory Factor
LPAM - Lymphocyte Peyer's Patch Adhesion Molecule
LPS - Lipopolysaccharides

M

MadCAM - Mucosal Addressin Cell Molecule
M-CSF - Macrophage Colony Stimulating Factor
MCP - Monocyte Chemoattractant Protein
MHC - Major Histocompatibility Complex
MIP - Macrophage Inflammatory Protein
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

NK - Natural Killer
NO - Nitric Oxide

O

OP - Optical Density

P

PBS - Phosphate Buffer Saline
PECAM - Platelet/Endothelial Cell Adhesion Molecule
PDGF - Platelet-Derived Growth Factor
PGA - Poly Glycolic Acid
PGE - Prostaglandin E
PHB - Polyhydroxybutyrate
PLLA - Poly -L-Lactic Acid
PMA - Phorbol-Myristate-Acetate
PMMA - Polymethylmethacrylate
PMN - Polymorphonuclear Neutrophil
PS - Polystyrene

R

RANTES - Regulated Upon Activation Normal T Cell Expressed and Secreted
ROS - Reactive Oxygen Species

S

SCA - Starch/Cellulose Acetate
SEM - Scanning Electron Microscopy
SEVA-C - Starch/Ethylene Vinyl Alcohol
SPCL - Starch/Polycaprolactone
SPLA70 Starch/Poly-Lactic Acid

T

THA - Total Hip Arthroplasty
Th - T helper
Tc - T Cytotoxic
TCPS - Tissue Culture Polystyrene

TCR - T Cell Receptor
TGF - Transforming Growth Factor
TNF- Tumour Necrosis Factor

V

VCAM - Vascular Cell Adhesion Molecule
VLA - Very Late Activation
Vn - Vitronectin

SECTION I

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 BIOCOMPATIBILITY – AN OVERVIEW

The performance of a medical device is controlled by two sets of characteristics, those which determine the ability of a device to perform the appropriate and specific function and those which determine the compatibility of the material within the body - biofunctionality and biocompatibility.[1] As such, the approach in the assessment of material biocompatibility encompasses the evaluation of the effects of physiological environments on materials and of the materials effects on the environment.[2]

The effect of the biomaterials on biological systems can be evaluated at different levels composing the consecutive stages of assessment. The assessment of the *in vitro* toxicity of a biomaterial is the initial step on a biocompatibility study, and is usually performed using immortalised cell lines[3,4] being often a qualitative analysis, based on the morphological examination of cell damage and growth when in direct[5,6] or indirect contact[7,8] with the materials. Toxicity involves the disturbance of cellular homeostasis[9] therefore affecting cellular functions that can be very subtle or lead to a multiplicity of biochemical changes.

The types of tests to determine potential adverse or toxic effects of medical devices include procedures designed to evaluate cytotoxicity, acute and chronic toxicity, irritation to skin, eyes and mucosal surfaces, sensitization, hemocompatibility, genotoxicity and carcinogenicity[10-12]. However, depending on the intended use of the devices, as well as the nature of their body contact, these general tests are not sufficient to demonstrate the appropriateness of the devices. Thus, the specific clinical application of the newly developed biomaterial determines which tests are further indicated.[3,13,14]

The emergence of new technologies in the development of biomaterials increases the need for adaptation of the existing standards.[10,11,15] For example, in the evaluation of the biocompatibility of biodegradable materials several variables, which were not considered for non-biodegradable materials, have emerged. In focus were the possible effects of the metabolites resulting from the degradation, the local and remote interactions of cells with those products and the rate and mechanism of degradation of the devices.[4,16-20] Considering that the results obtained with the standard tests could be influenced by those new parameters, adaptations have been made along the way. Long term tests were initially considered only *in vivo* but the study of degradable materials has to predict in vitro, as much as possible, the continuous effect of those systems and mainly of their degradation products.[4,16-20] Of course it is not possible to maintain in vitro cell cultures for indefinite time thus, the need to mimic long-term degradation, launched biocompatibility tests using extracts

of the materials obtained under different conditions.[15-17,19] High temperature degradation assays are based on the assumption that the degradation of biodegradable materials can be accelerated at high temperatures thereby releasing products that are expected to be released in vivo after long-term implantation.[15,21] However, there is still some controversy about the veracity of these high temperatures having the same influence on the degradation behaviour to what might occur in vivo. The so called "real-time degradation tests" were suggested by International Standard Organisation (ISO) standards.[15,17,19] Extracts are obtained at 37°C, body temperature, at different times which can go up to 52 weeks. Furthermore, since the human body is a dynamic system with constant changes of fluids, the simulation of the degradation of the materials under movement/shaking was also considered as a way of simulate better the in vivo conditions.[15]

Generally the biocompatibility results obtained for biodegradable materials using the current standards are not the most promising ones. The pH and osmolarity of polymer extracts have been suggested to be related to the toxicity of polymers[20,22] and dependent on the amounts of solubilised monomers and oligomers[23]. In fact, pH influences cell behaviour and viability and acidic ph lower than the physical pH of the cells can cause a toxic response[24,25]. In addition, osmolarity is another factor that can exert an influence on proliferation, morphology and cell activity[26]. We might suggest that the key for overcoming these issues could be the control of the kinetics of degradation of the biodegradable polymers.

1.1.1 *In Vitro* Testing

The need to restrict animal experimentation to a minimum enhanced the necessity to use *in vitro* systems to select, adequately, potential useful biomaterials and those unsuitable for human application. Using cell lines to perform cytotoxicity screening has become routine in almost all laboratories around the world. However it must be stressed that in many cases the ideal situation is the use of human, non-transformed cells that is, primary isolated cells in early passage.[10-12,27] Both approaches have advantages and disadvantages thus, many authors[4,10-12,27,28] defend the use of cell lines at the first stage of screening and the use of adequate primary cultures relevant to the purpose for which the potential biomaterial has been developed.

Primary cell cultures are achieved through enzymatic or mechanical disaggregation of a piece of tissue or by spontaneous migration from an explant and may be propagated as an adherent monolayer or as a cell suspension.[29,30] These cells are generally heterogeneous, with a low fraction of growing cells but with a variety of cell types representative of the tissue.[29-31] The major advantage is their ability to proliferate and replicate, although specialised cells and functions can be lost with the propagation of the culture.[29-31]

Cell lines are previously established, and generally have origin in transformed primary cultures. While these have limited life, a continuous cell line is immortal and may be derived from different species and tissues.[30] Comparatively with primary cultures of the same type of cells, a cell line presents morphological alterations such as decrease cell size, reduced adherence and higher nucleus.[11,31,32] The tumour nature of these cells theoretically involves the possibility of phenotype and genotype variations.[31] The main drawback using cell lines can be the extrapolation of results obtained in vitro to in vivo situations where normal cells act.[4,27,28] Cell lines have however the advantages of being highly sensitive and homogeneous allowing for it use for a long time period representing an abundant source of cell material.[28,30] Both cell types can be frozen and retrieved intact, even after many years. Within cellular phenomena, biocompatibility testing gives high importance to cell death, cell proliferation, cell morphology and cell adhesion, which directly correlate with toxicity in vitro.[3,9,31]

Several methods[4,7,13,14,16,27,33,34]have been used to quantify cell proliferation mainly based in the quantification of total protein[7,27,33]or DNA[4,13,14,16,27,34] and in the measurement of DNA synthesis following the incorporation of radiolabelled nucleotides[4,16,27,35,36]. These molecules are taken up into DNA of cells during mitosis allowing for the identification of new cells by auto-radiography. The synthesis of DNA can then be followed, through the incorporation of labelled bromodeoxyuridine (BrdU)[4,16], a pyridine analogue, and ^{3}H- Thymidine[27,35,36]. In the case of BrdU, monoclonal antibodies coupled with a visualization system such as peroxidase-antiperoxidase were also used avoiding then the radioactive precursors.[37] A growth curve can be obtained by counting the number of cells in cultivation at different intervals after seeding which allow to extrapolate a cell number from the cytotoxicity test.[16] This usually involved enzymatic treatment to obtain a cell suspension which is then counted.

Loss of viability constitutes the critical consequence of a toxic biomaterial. A qualitative evaluation of cell morphology is often based on the examination by inverted microscopy. Typical characteristics of cytotoxic cellular alterations include shrinking of the cell nucleus, fragmentation of the cytoplasm, granulation formation, rounding off and cell detachment.[16,29,32] Furthermore, a reduced biosynthetic activity[16] as well as the release of cytoplasmic metabolites[38] or uptake of non-viable stains[39], resulting from cell membrane rupture, might be indicators of cell death. Several methodologies, based on these indicators, have been used to quantify cell viability. An example of cell stain is neutral red[13] which is endocytosed by viable cells and internalised inside lysosomes allowing for conclude about cell integrity. Contrarily, trypan blue is taken up by cellular proteins within nonviable cells and excluded by living cells.[13,14,30] The stains can also be measured spectrophotometrically and compared with a standard curve for cell quantification.[14] A combination of the two principles was suggested by Dankberg et al[40] using a combination of fluorescein diacetate (FDA) and ethidium bromide (EB). FDA is taken up by intact cells and converted by esterases to the

polar compound fluorescein, which remains in the cytoplasm giving green fluorescence on UV-excitation. EB can only penetrate cells with damaged plasma membranes and binds to nuclei acids resulting in an orange-red fluorescence. Others[14] have used acridine orange (AO) instead of FDA.

Cell death has also been quantified after contact with biomaterials by a tetrazolium-based colorimetric assay which uses a yellow dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduced, by the mitochondrial succinate dehydrogenase of alive cells, into a purple formazan salt.[4,16,27,41]

The membrane integrity, therefore cell viability, can also be evaluated by measuring lactate dehydrogenase activity (LDH).[20,38,42] This cytosolic enzyme has been used for many years to measure the cell membrane damage. Indirect measurement of LDH activity, which is present in the cytoplasm of intact cells, can occur only if cells are lysed and since LDH activity was proved to be directly proportional to the number, of viable cells can be determined.[20,38,42]

In addition to the evaluation of cell morphology and viability the use of cell dyes, enable cells adhesion behaviour to be observed. Cell attachment represents the translation of certain physico-chemical events involving the chemical interaction between cells and materials.[43] This is followed by cell adhesion which is the result of biological processes such as the production of extracellular matrix proteins and cytoskeleton protein reorganisation amongst others.[43] Cell adhesion and spreading were shown to be clearly distinguishable biological phenomena because substrates that allow cell adhesion do not necessary promote cell spreading[14]. Furthermore, it was previously suggested that surfaces that show good cell attachment at early time points do not necessarily promote cell proliferation or differentiation.[44] After cells contact surfaces, cells will alter their cell membrane and morphology to stabilise the cell-material interface.[45] Filopodia, finger-like protrusions of plasma membrane formed as a consequence of actin assembling in long bundles or lamellipodia if assembled in the form of mesh supporting sheet-like protrusions are morphological details, characteristic of cell adhesion.[43] When cell adhesion was followed by progressive flattening of the cells, proliferation occurred.[46] Analysis of results should be cautious however. In hostile environments, anchorage-dependent cells become round, detach from the substratum and die.[47] Nonetheless, reduction of cell adhesion may be wrongly interpreted since it may not be a toxic effect and has to be distinguished from cell death. If certain surfaces aim to be adhesive for cells working as a substrate for cell proliferation others will be biocompatible if cells fail to adhere to their surface or to the tissue culture polystyrene plates in the presence of their extracts.[7,14,16] This allied to a morphological evaluation of the cells allow to confirm the reduced cell adhesion as a signal of toxicity.

At this point of evaluation of biocompatibility, it is extremely important to proceed mimicking, as much as possible, the environment that the implant will face thus using cells related to the function for which they were designed.[3,13,14]

In orthopeadic applications osteoblast adhesion and direct apposition of bone matrix to the implant surface are mandatory in the development and maintenance of the bone-implant interface. Several studies[48-58] involving osteoblast adhesion have been performed in order to try to understand the cellular events that occur at the osteoblast-implant interface.

Surface characteristics of the materials, whether their topography[53,56], chemistry[56,59-61] or surface energy[49,52], play an essential part in cell adhesion on biomaterials. Attachment, adhesion and spreading belong to the first phase of cell/material interaction and the quality of this stage influences the capacity of cells to proliferate and differentiate themselves on contact with the implant.[43]

Chemical properties such as carboxyl[62] and hydroxyl[58,63,64] groups can be important in cell attachment and growth depending on the type of cell. For instance, Curtis et al[63] found that the blocking of hydroxyl groups results in lost cell adhesion but Horbett et al[65] reported that the presence of an excessive number of OH groups has an opposite effect. These studies suggest the need of an optimal density for OH groups in order to obtain good cell adhesion. In addition others have reported[55] that OH groups are responsible for higher surface polarity and hydrophilicity of the surface, but a correlation between these two parameters has not been determined. Some authors[66-68] defend that cell adhesion is generally better on hydrophilic surfaces, while other studies[50,51,55] showed that osteoblast-like cells do not display a consistent trend of behaviour in relation to surface wettability but rather varied as a function of particular functional groups. Wettability was also related to protein adsorption. Cell attachment was shown[69] to be preferred on moderate hydrophilic surfaces and it has been suggested that these surfaces permit the adsorption of serum proteins with labile and reversible bonds. The moderate degree of wettability of the substrates allows cells to deposit their own adhesion proteins, exchanging with the more rapidly adsorbed serum proteins. This mechanism was proposed[70] to be slower on extremely hydrophobic or hydrophilic surfaces, justifying the fact that cells do not adhere and proliferate so well on those surfaces. Nonetheless other work with osteoblasts suggested that cell adhesion was greatly influenced by the polar interaction energy[49,52], which emphasises the role of surface energy in this biological process.

In addition to chemistry, osteoblasts react differently according to surface topography[53,56] and roughness[48,54,57,58]. Rougher surfaces were shown to reduce proliferation of osteoblast-like[48,54] and human bone derived cells[57,71]. In addition, the initial adhesion of osteoblast-like cells was shown to be greater on polished (smoother) surfaces.[54]

A direct relationship between roughness and surface energy of the materials does exist and it was demonstrated that the apolar component of surface energy increased significantly with roughness.[72] Furthermore, it was reported[73] that for relatively low surface roughness values, cell responses to the surface chemistry are more important than the physical surface.

Some studies[50,55,57,74] demonstrated ultrastructural differences in cell spreading and filopodia forming on a surface even if no differences in the percentage of adherent cells were observed[74].

Morphological aspects have also been shown to be influenced by different chemistries.[50,55,56,66] A critical value for the surface energy of the substratum above which cell spreading occurs, was previously established.[75] Likewise, cytoskeleton organisation and cell morphology are regulated by surface wettability[49,50]. Cell attachment and spreading are generally greater on certain moderately hydrophilic surfaces relative to hydrophobic ones[49,50]. Osteoblasts are also shown to recognise substrate morphology and to respond by altering their degree of spreading[76,77]. Several studies[54,57] have demonstrated that cell spreading and continuous cell layer formation was better on smooth surfaces compared to rough ones. However, Bigerelle et al[78] suggested that topography below the cell scale favours polygonal morphology of osteoblasts although when the topography was considered above the cell scale they also appreciate the roughness may explain cells being spread and flattened on surfaces considered rough[76].

1.1.2 *In Vivo* Testing

Several implantation procedures have been suggested in order to obtain the most adequate system for each type of material for the evaluation of local toxicity. It is important to evidence that besides the materials, there are other issues related with the surgical technique that influence quite extensively the host response.[15] Thus, although the most currently used intramuscular and subcutaneous models represent appropriate choices, new options are always under study aiming to reduce as much as possible the interference of other variables apart from the implant.[19,28,79]

Degradable materials, which will stay in the human body long-term, will induce not only local but also systemic effects.[79-81] The degradation products, their concentration, the changes occurred in the materials such as alteration of the shape, roughness as well as for example the mechanical irritation of the more degraded materials in the biological tissues, may induce local and remote interactions between material/products and the biological system.[20,82-84]

Furthermore, the evaluation of the implants *in vivo* performance can be achieved by means of biomechanical testing. After sacrificing the animal, shear strength, bending and tensile

tests are commonly performed to compare device characteristics before and after implantation.[85]

An acute phase follows the implantation of any material the severity and extent of which depends on several parameters mainly related with the material. If the deleterious action of the materials remains, a chronic response persists, leading in many cases to the failure of the implant.[83,84] Both acute and chronic reactions can persevere for many years.

The assessment of the type and extension of response that a biomaterial might locally induce is based on the histological analysis of the tissues surrounding the implant. Several factors have been taken into account within that analysis helping to define the degree of toxicity of the implant. The presence and amount of certain types of cells such as neutrophils, monocytes, macrophages, eosinophils, lymphocytes, fibroblasts and foreign body giant cells at the interface tissue-material are indicative of the response elicited by the implant.[79,83,84] Immuncytochemistry techniques are often used to identify the cells present in the retrieved implant as well as in the local tissues and complemented with image analysis systems to quantify the number of cells and their distribution related to the implant.[79,86] Many of these cells when activated, produce hydrolytic enzymes responsible for the lesion of the tissues thus, the enzymatic activity in the tissues surrounding the implant can be determined in order to identify the presence or not of the toxic stimulus.[87,88]

Following the implantation of any medical device, the wound healing mechanisms are triggered in response to injury and to the presence of a foreign body.[89,90] Thus the host generate a response aiming to eliminate the cause of injury and to repair the damaged tissues. The formation of a fibrous capsule is a common occurrence after the implantation of biomaterials but its formation depends on the severity of the response induced by the implant and consequently on its properties.[20,82-84] Thus, the thickness of the fibrous capsule formed around an implant was also suggested[20,83,84,89,90] to be a measure of the toxicity of the materials studied.

Because of the interconnection between tissues and organs through blood, the lymphatic system and interstitial tissue fluid, exchange of products takes place between the implantation site and the rest of the body. Remote site effects are slightly neglected within the in vivo biocompatibility evaluation of potential biomaterials. Rather than histological analysis, the assessment of systemic effects can be done examining physical symptoms like hypokinesia, dyspnea, diarrhea, cyanosis, tremors and, the worst scenario, eventually death.[79-81,91]

1.2. IMMUNOCOMPATIBILITY

Many materials are proposed or currently used in a wide range of biomedical applications[92-96] but few are considered ideal and most of them can be hypothesised as foreign by the host and prompt a tissue response[82,84,97,98]. In general these biomaterials do not exhibit comparable physical, chemical or biological properties to natural tissues and ultimately, these devices can lead to chronic inflammation and foreign body reactions[82,98-100].

To date, a complete understanding of the biological responses to implanted biomaterials is still missing. The mechanisms of how a body reacts to implants over the course of time by inflammation, wound healing and the foreign body response is not fully understood. Immune system cells and chemical mediators are thought to be very important players in those reactions and will be present at the implantation site, independently of the function of the device. Thus the evaluation of the mechanisms of inflammation, wound healing and foreign body reactions may provide useful information about the immunocompatibility of newly developed biomaterials.

The implantation of a biomaterial into human tissues triggers a set of cellular and biochemical processes collectively known as inflammation, in response to the injury and to the presence of the implant[82,101,102]. Leukocytes have been identified as the main cell types responsible for the adverse reactions implicated in inflammation.[99,103,104] Severe and persistent leukocyte activation may lead to compromising alterations in the function of an implant and eventually to the failure of the device. In addition, there is some controversy about which properties of the surface of materials stimulate particular cell/tissue reactions. It has been hypothesized that not only the wettability and surface charge of the surface of the materials, but also the presence of certain functional groups have importance for the adhesion and activation of immunological cells in vitro[105-112]. Furthermore, the degradation rate and mechanisms of degradation in biodegradable devices can also modulate[113-116] and might allow control of tissue responses in vivo.

The acute inflammatory response is immediately initiated, leading to exudation of plasma proteins and inflammatory cells that migrate to the site of injury[117]. During this early stage several chemical mediators control and determine the extent of the reaction which would culminate in the restoration of the tissue and consequently wound healing[118]. However, it is becoming clear that in the presence of an implant the normal healing of injured tissues does not necessarily occur via the same mechanisms[119]. A chronic inflammatory response is often instigated and can be maintained or amplified, depending on the material properties, causing damage of the host tissues[120-122]. This process is mainly controlled by chemical mediators known as cytokines; these substances are produced by the cells present at the implantation site and can act locally or systemically attracting other cells and inducing the production of

other cytokines as well as guiding cellular functions.[123] The so called cytokine network represents a very complex system of many molecules with multiple actions, involving many different types of cells and intermediaries[124].

1.2.1 Immune System

Immunity means ability of an organism to resist disease by identifying and destroying foreign substances or organisms[125,126]. Cells and molecules involved in such mechanisms constitute the immune system and the response resulting from the introduction of a foreign agent is known as the immune response. However, not all immune responses occur to protect the host from disease; there are situations, although not the majority, when sickness is caused by an immune reaction[127-131]. These are for example allergic reactions, which occur due to the presence of external stimuli or autoimmune diseases such as multiple sclerosis[130] or rheumatoid arthritis[129,131], where an individual reacts against their own tissues. The implantation of any medical device can be considered an external invading element that might induce an immune response, mostly but not exclusively, dependent on the properties of the device itself[101,105-107,132-134].

Central organs of the immune system include the bone marrow and thymus, which are involved in generating precursor lymphocytes rather than immune responses[126,135]. In addition to lymphocytes, monocytes and granulocytes derived from precursor stem cells in the bone marrow[126]. The lymph nodes and spleen are known as peripheral organs and have as main roles to optimise interaction between antigen presenting cells (APC) and T and B lymphocytes[135].

Lymphocytes have receptors for antigen and confer specificity to an immune response. These cells express receptors with varying affinity for the antigen in question. Thus, during lymphocyte development the cell with the highest affinity for the most abundant antigen will have a growth advantage and will preferentially generate progeny of itself[135].

There are two types of lymphocytes, B and T; B cells have their origin in the bone marrow of adult mammals, whereas T lymphocytes undergo further maturation in the thymus. B lymphocytes migrate directly from marrow to peripheral lymphoid tissue producing antibodies and some soluble mediators called cytokines[124]. On the contrary, T lymphocytes do not produce antibody molecules but have surface receptors structurally related to Immunoglobulins (Ig)[136].

Once released from the bone marrow and thymus, lymphocytes begin to populate the whole lymphoid system. The higher concentration and the degree of accessibility of these cells, enables a rapid response to infectious agents.

T cells see antigen by recognising peptide fragments complexed with surface Major Histocompatibility Complex (MHC) glycoproteins on neighbouring cells. The cell surface glycoproteins encoded by genes in the MHC allele, bind fragments of antigen after it has been subjected to antigen processing[137].

There are two sub-sets of T cell divided according to their function; T helper (Th) and T cytotoxic (Tc) both are involved in cytokine production, but they also have individual actions, respectively helping B cells and T cytotoxic cells in the lysis of infected and tumour cells[138]. Surface proteins expressed by the different cells of the immune system, have been given standardised names, characterised by the initials CD (Cluster Designation) and a number. CD4 and CD8 are specific for the two T cell subsets, but the CD markers can be specific for individual populations of cells, or particular phases of cellular differentiation or activation. Th cells express CD4 and present antigens in association with MHC class II molecules while Tc lymphocytes express CD8 and present antigen using MHC class I proteins[139].

B cells use a different mechanism of antigen presentation; after binding to a cell surface antibody their specific antigen is internalised, partially degraded and presented to Th cells in association to MHCII molecules[140].

Natural killer (NK) cells are large granular lymphocytes that are cytotoxic in the absence of prior stimulation. NK cells represent a first line of defence to infections, tumour growth and other pathogenic alterations of tissue homoeostasis possessing receptor molecules which allow them to detect some infected host cells, including tumour cells, virus, or intracellular bacteria-infected cells[138]. NK cells do not express antibodies or T cell receptors at their cell surface, but produce cytokines and express receptors for immunoglobulins[141].

Phagocytic cells, also part of the immune system, are critical in the defence against bacterial and simple eukaryotic pathogens. Mononuclear phagocytes, in particular monocytes/macrophages, can recognise bacterial and yeast cell walls through broadly specific receptors, usually for carbohydrate structures, being able to take them up by phagocytosis[142]. Besides monocytes/macrophages, mononuclear phagocytic cells also include microglial cells in the central nervous system, endothelial cells of vascular sinusoids and reticular cells of lymphoid organs, which take up large particulate antigens, pieces of tissue, senescent cells and bacteria [138].

Mononuclear phagocytes have important properties; they express a myeloid receptor (CD14) which serves as a recognition molecule for a wide variety of bacterial envelope molecules, such as lipopolysaccharides (LPS), which, after interaction lead to macrophage activation[143]. Mononuclear phagocytes can act as APCs for T cells and at the same time can be activated by T cell derived cytokines leading to increased phagocytosis and microbicidal activity (increased activity of degradative enzymes, prostaglandins, nitrogen and oxygen free radical production)[144]. Furthermore, these cytokines also increase the antigen presenting activity of

macrophages, which, in turn, are able to present antigen to T cells. This cycle will continue as a positive feedback loop until the antigen is eliminated[145].

Mononuclear phagocytes express receptors for antibodies and complement, which means that they bind immune complexes, especially if the antibody involved has complement components bound to it, in which case cells endocytose/phagocytose these rapidly[146].

Other APC are the group of dendritic cells; these are of two types and although having similar names they have different functions. Cells of the dendritic cell (DC) lineage are bone marrow derived but they are also present in the skin where they are known as Langerhan Cells (LC)[138]. These cells efficiently process antigen but cannot present it to T cells. LC pick up antigen in the skin and carry it via afferent lymphatic vessels to lymph nodes. Here, the tissue dendritic cells or interdigitating cells, may efficiently present antigen if they encounter the right T cell. In fact these are the most efficient APC, since far fewer DC are required to initiate an immune response than any other APC[147].

Granulocytes constitute another class of immunological cells. There are three types of granulocytes: neutrophils, eosinophils and basophils. Neutrophils, also known as polymorphonuclear leukocytes, they express receptors for immunoglobulin and complement and are involved in the acute inflammatory response[148]. Eosinophils carry receptors for immunoglobulin E (IgE) and are involved in the destruction of IgE coated parasites and contribute to the response to allergens[149]. Basophils are the circulating counterpart of tissue mast cells. They express high affinity receptors for IgE and are stimulated to secrete the chemicals responsible for immediate hypersensitivity following antigen induced aggregation of these receptors[149].

Each type of cell of the immune system experiences a series of processes which involve not only other types of cells but also chemical mediators that are enrolled in its regulation. These characterize the immune responses. It is possible to make a distinction between innate and adaptive immunity respectively as the capacity to respond to foreign agent instinctively or in a specific manner. The innate or natural response is present in neonatal animals while in the case of adaptive response the immune system requires pre activation and memory towards the foreign agent. Natural immunity depends on a variety of immunological effector mechanisms which are neither specific nor improved by repeated encounters[138]. Among others, its components are the complement system, acute phase proteins and interferons. In turn, the adaptive immunity increases in effectiveness and strength each time the host contacts the invaders[125,138]. This constitutes a useful evolutionary adaptation because it improves the efficacy of the innate immune response by focusing the response to the site of invasion/infection as well as providing additional effector mechanisms that are unique to lymphocytes[125,150]. The difference between innate and acquired immunity lies in the antigen

specificity of lymphocytes[125]. This property is conferred upon lymphocytes by the expression of cell surface receptors that recognise discrete parts of the antigen.

From a different perspective, it is possible to organize the immune response in a dual system known as cell-mediated immunity and humoral immunity. Both systems are adaptive and respond specifically to most foreign substances although, depending on the antigen, one immune response generally is favoured over the other[151]. Lymphocytes are involved in both types of reactions. T cells are responsible for the cellular immunity because they are directly involved in the response, and B lymphocytes are implicated in the humoral response reacting with the antigen and producing antigen specific antibodies[125,138]. An antigen is then defined as any substance that can bind to a specific antibody and comprises an enormous range of substances from simple chemicals, sugars and small peptides to complex protein complexes such as viruses[125].

During an immune response, a complex lattice of interlinked antigens and antibodies, known as an immune complex, will present an array of constant regions, which can activate cells through the binding of their immunoglobulin receptors. Antibodies can act in different ways; by blocking the biological activity of their target molecule e.g. an enzyme binding to its receptor; neutralisation, interacting with special receptors on various cells, including macrophages, neutrophils, basophils and mast cells allowing them to recognise and respond to the antigen; opsonisation, and causing direct lysis by complement which also enhances phagocytosis; complement activation[125].

1.2.2 Adhesion Molecules

Cell adhesion molecules (CAMs) play an essential role in adhering circulating leukocytes to the vascular endothelium at the sites of inflammation and then their subsequent transmigration into adjacent tissues. In the absence of signals to stimulate the expression of CAMs, the adhesive forces between endothelium and leukocytes are not enough to attach leukocytes.[152]

The adhesion molecules can be divided into three families of different structural architecture: selectins, integrins and certain glycoproteins included in the Ig superfamily[153].

Cytokines have been implicated in the up-regulation of many CAMs, this expression increases cell adhesion between leukocytes and endothelium, which may be crucial to the regulation of inflammatory processes[154]. Cytokine-activated endothelial cells also secrete chemokines such as interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 required for leukocyte recruitment[155].

Selectins are molecules which mediate leukocytes and platelets to vascular surfaces, they are characterised by an extracellular motif involving two domains: a lectin-like domain

attached to an epidermal growth factor (EGF) like domain and a variable number of complement regulatory protein repeat sequences[153,156-158].

There are three selectins which have been shown[159] to be important in the cell to cell adhesion process; L-selectins are constitutively expressed on leukocytes while E-selectins are present exclusively in endothelial cells, being only expressed following stimulation by cytokines. In contrast P-selectins are accumulated pre-formed for rapid release in platelets or endothelial cells[160].

Selectins play a critical role in the leukocytes initial attachment and rolling on the vascular endothelium prior to integrin action[161]. Endothelium becomes activated by inflammation-induced cytokines, in turn resulting in the expression of selectins[153]. The interaction of P- and E- selectins with the carbohydrate ligands on the surface of leukocytes appears to be responsible for initiating their rolling on the endothelium[153]. Although neutrophils and some lymphocytes constitutively express L-selectin, it is only after E- and P- selectin expression by endothelial cells that the rolling process occurs[158,161].

Integrins are another class of adhesion molecules; they are heterodimers consisting of non-covalently linked α and β subunits. There are many possible combinations between the different known subunits, however, β_2, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins are the main intervenients in regulating immune cell adhesion to endothelium[153].

Within the β_2 integrins, it is possible to find the surface antigen expressed in all leukocytes, known as lymphocyte function-related antigen (LFA-1); it is an integrin consisting of a α subunit (designated CD11a), and the β_2 subunit (designated CD18)[152]. The α subunit can vary in the heterodimer region, originating two other important adhesion molecules, the Mac-1 (designated CD11b) and C3b receptor (designated CD11c), both are expressed on monocytes/macrophages and granulocytess[162] but not on lymphocytes[163]. CD11a is involved in the adhesion of leukocytes to endothelium and Mac-1 plays a key role in the adherence of both monocytes and neutrophils to vascular endothelium for subsequent extravasation[164,165]. CD11b/CD18 is also implicated in a variety of cell-cell and cell-substrate interactions such as attachment and phagocytosis of particles coated with C3bi by granulocytes and macrophages[166].

The α_4 subunit-containing integrins have been termed VLA (very late activation) since two of them are expressed on lymphocytes about two weeks after antigen stimulation *in vitro*[166]. VLA-4 ($\alpha_4\beta_1$; CD49d:CD29) expressed in resting lymphocytes and monocytes is probably the most important VLA integrin with respect to cell adhesion[152].

The $\alpha_4\beta_7$ integrin, also known as lymphocyte Peyer's patch adhesion molecule-1 (LPAM-1), is expressed on the microvillus tips of lymphocytes mediating the adhesion to the walls of inflamed venules[153].

It is important to emphasise that the adhesion and consequently the cell migration to a site of injury via integrins is dependent on the changes which occur in the adhesion molecules subunits. Rather than an increase in the amount of expressed CAMs, stimuli like cytokines and antigens induce a conformational change of the integrins from a low to a high affinity state[153].

In addition to their role in the adhesion of cells to endothelium, integrin receptors on the neutrophil cell surface facilitate binding of neutrophils to the extracellular matrix[167].

Membrane proteins belonging to the Ig superfamily are specific cell surface molecules which act as counter-ligands for integrins. Those which are expressed on endothelial cells can be also designated as Ig-like addressins[152].

Some representative examples of membrane proteins members of the Ig superfamily are: Ig-α/Ig-β heterodimer, part of B cell receptor, T cell receptor (TCR), T cell accessory proteins such as CD2, CD4, CD8, CD28 and the γ, δ, and ε chains of CD3, class I and class II MHC molecules, platelet-derived growth factor (PDGF) and various cell-adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), ICAM-2 and LFA-3.[124]

1.2.3 Cytokines

Cytokines are local protein mediators involved in almost all important biological processes namely, cell growth and activation, inflammation, immunity and differentiation[168]. Molecularly, cytokines are defined as inducible, water soluble, heterogeneous, proteinaceous mediators, possessing specific effects in target cells and/or in the mediator-producing cells themselves[169]. Cytokines exert their effects by binding to specific cell-surface receptors which signal to their target cells[170]. They act at very low concentrations (typically 10^{-10} to 10^{-12} M), are short-lived and may act, either on other cells (paracrine) or on the same cell (autocrine), or systemically (endocrine)[124].

The term cytokine encompasses different classes, interleukins which refer to a group of cytokines which are typically produced by T lymphocytes and macrophages although other leukocytes are also able to secrete them in lower amounts[168]. Another group of cytokines is designated by the generic name of chemokines due to their effects in the chemotaxis of leukocytes[124].

However, it is often not clear which molecules should be defined as cytokines, particularly in the case of hormones and growth factors (GF), but the pleiotropic nature of cytokines enabled the problem to be clarified[168]. Furthermore, GF tend to be produced constitutively, whereas cytokine production is carefully regulated and, unlike hormones, which act long

range in an endocrine way, most cytokines act over a short distance in an autocrine or paracrine manner.[124]

The cytokines known collectively as pro-inflammatory cytokines stimulate or accelerate inflammation and also regulate inflammatory reactions either directly or by their ability to induce the synthesis of cellular adhesion molecules or other cytokines in certain cell types[169]. The major pro-inflammatory cytokines that are responsible for early acute phase responses are IL-1α, IL-1β, IL-6, and tumour necrosis factor-α (TNF-α). Other pro-inflammatory mediators include interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), granulocyte and macrophage colony stimulating factor (GM-CSF), leukaemia inhibitory factor (LIF), and the interleukins IL-8, IL-11, IL-12, IL-17, IL-18[169,171].

IL-1 is a major mediator of inflammation and in general initiates and/or increases a wide variety of non-structural, function associated genes characteristically expressed during inflammation[172]. Although secreted by a variety of cells such as activated macrophages from different sources (alveolar macrophages, Kupffer cells, adherent spleen and peritoneal macrophages), peripheral neutrophil granulocytes, endothelial cells, fibroblasts, smooth muscle cells, keratinocytes, langerhans cells of the skin, osteoclasts, astrocytes, epithelial cells of the thymus and the cornea, T cells, B cells, and NK-cells, monocytes and tissue macrophages are the main source of IL-1[124,172,173].

There are two functionally almost equivalent forms of IL-1, IL-1α and IL-1β that are encoded by two different genes[174]. IL-1β is the predominant form in humans, while IL-1α is found more abundantly in mice[169,175]. Mature forms of IL-1α and IL-1β and also their precursors are secreted by murine macrophages after stimulation with bacteria or numerous microbial products[176]. Both forms of IL-1 bind to the same receptor and therefore also show similar if not identical biological activities[177] with only a few functional differences between the factors having been described.

Within a few minutes of binding to cells, IL-1 induces several biochemical events. This cytokine is strongly involved in the proliferation mechanisms of several cells acting as a stimulant for NK-cells and fibroblasts and as an inhibitor for endothelial cells. IL-1 causes many alterations of endothelial functions *in vivo*. It promotes thrombotic processes and attenuates anticoagulatory mechanisms. IL-1 therefore plays an important role in pathological processes such as venous thrombosis, arteriosclerosis, vasculitis, and disseminated intravasal coagulation[178]. Chemotactic properties are also attributed to IL-1; it is a strong chemoattractant for leukocytes in particular neutrophils.

TNF-α, another pro-inflammatory cytokine, is produced by activated mononuclear phagocytes, macrophages and lymphocytes as well as many other non-immune cell types[179]. TNF-α is particularly important in organising reversible microenvironments, and its production

can induce remarkable cellular changes and tissue remodelling[180]. Like IL-1, TNF is a potent activator of neutrophils, mediating adherence, chemotaxis, degranulation and respiratory burst[181]. However, this cytokine has paradoxical roles in the inflammatory process. While inducing death of diseased cells at the site of inflammation, this cytokines stimulates fibroblast growth[182,183]. In the skeletal system, TNF-α stimulates bone and cartilage resorption and inhibits proteoglycan and collagen synthesis under some conditions[184].

Together with IL-1, IL-6 is a major physiological mediator of the acute phase reaction, inducing hepatic expression of acute phase proteins[185,186]. It is produced by many different cell types but the most important source is mononuclear phagocytic cells[187]. Macrophages, T cells and B lymphocytes, granulocytes, smooth muscle cells, eosinophils, chondrocytes, osteoblasts, mast cells, glial cells, and keratinocytes also produce IL-6 after stimulation[124,179].

IL-6 has pleiotropic functions influencing antigen-specific immune responses and inflammatory reactions. IL-6 is a B cell differentiation factor in vivo and in vitro and an activation factor for T cells[188]. However, in contrast to those pro-inflammatory effects, IL-6 also possesses anti-inflammatory functions namely inhibition of IL-1, TNF synthesis[189] and stimulation of IL-1 receptors antagonist (IL-1ra) production.

The most important cytokine responsible for cell-mediated immunity is IFN-γ[190]. The expression of IFN-γ was long considered to be restricted to activated T and NK cells[191]. The production of this cytokine requires activation of the cells, which can occur via a combination of different types of signals; a specific or non-specific ligand interaction with a T cell receptor, their contact with accessory cells through adhesion molecules, and by a combination of cytokines[192]. Although initially considered to have antiviral functions, it has become clear that IFN-γ has a broader role[191]. Compared to other interferons, IFN-γ growth inhibitory activities are more pronounced and its main biological activity appears to be immunomodulatory in contrast to the other interferons, which are mainly antiviral.

As the majority of cytokines, IFN-γ is seen as a pro-inflammatory cytokine with a pleiotropic nature mainly to increase TNF activity and nitric oxide (NO) secretion[193] and to activate the pathways that lead to cytotoxic T cells[194]. IFN-γ exerts important activities on both monocytes/macrophages and lymphocytes, which generally result in macrophage activation and T cell differentiation towards a T_H-1 type of immune response[194]. In addition, it can destroy blood vessels but also induce several angiogenic factors. IFN-γ inhibits the proliferation of endothelial cells and the synthesis of collagens by myofibroblasts, thus functioning as an inhibitor of capillary growth mediated by myofibroblasts and fibroblast growth factor (FGF) and PDGF.

Besides IFN-γ, another significant lymphocyte-derived interleukin is IL-2, which is produced mainly by T_h cells, expressing the surface antigen CD4, following activation by mitogen or

allogen. Several secondary signals are required for maximal expression of IL-2 and resting cells do not produce IL-2.[179,195] IL-2 induces proliferation of T lymphocytes however, this only occurs when IL-2 and IL-2 receptors (IL-2r) are simultaneously produced and expressed. Therefore, IL-2 is an antigen-specific proliferation factor for T cells ensuring that only the T cells specific for the antigen provoking the immune response become proliferative[179]. Due to its effects on T cells and B cells, IL-2 is a central regulator of immune responses.

Anti-inflammatory cytokines, contrarily to pro-inflammatory ones, are generally considered as possessing immunoregulatory and inhibitory properties. These mediators act mainly by the inhibition of the production of pro-inflammatory cytokines or by counteracting the many biological effects of pro-inflammatory mediators in different ways[169]. The main anti-inflammatory cytokines include[124]: IL-4, IL-10 and IL-13 but other anti-inflammatory mediators include IL-16, IFN-α, TGF-β, IL-1ra, granulocyte colony stimulating factor (G-CSF), as well as soluble receptors for TNF or IL-6. Although IL-4, IL-10 and IL-13 are considered anti-inflammatory cytokines due to their ability to suppress production of IL-1, TNF and chemokines, they are potent activators of B lymphocytes[169].

IL-4 is produced mainly by a subpopulation of activated T cells (T_h2) which are the biologically most active helper cells for B cells[124,196]. It promotes the proliferation and differentiation of activated B cells[197], and the expression of low affinity IgE receptors in resting B cells[198]. This cytokine can promote their capacity to respond to other B cell stimuli and to present antigens for T cells. This may be one way to promote the clonal expansion of specific B cells and the immune system may thus be able to respond to very low concentrations of antigens.[199]

The anti-inflammatory properties of IL-4 appear to be mediated at multiple levels, directly suppressing the production of pro-inflammatory cytokines[200,201], as well as antagonising the pro-inflammatory effects of IFN-γ on several functions[202,203].

IL-10 is secreted by both T_h1 and T_h2 cells[204], the major source in humans are monocytes and B cells[179] having the macrophage as it's main target. IL-10 suppresses cytokine production by macrophages, thus indirectly reducing cytokine production by T_h1 cells[179]. Furthermore it down-regulates the expression of MHC class II molecules in antigen-presenting cells[179,205].

IL-13 is homologous to IL-4 and shares a large amount of its biological activities on mononuclear phagocytic cells, endothelial cells and B cells[206]. This cytokine is however, more widely produced than IL-4 including by T_h1 lymphocytes being readily identified in allergic inflammatory tissue[207].

Chemotactic cytokines are a superfamily of low molecular weight proteins that facilitate the passage of leukocytes from the circulation into the tissues and are generally identified as chemokines.[208] These molecules are capable of inducing chemotaxis in a variety of cells

including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts and keratinocytes[179]. Despite being considered mainly as a chemotactic molecule their role is much more complex and it goes from recruiting and activating leukocytes to homeostatic functions[179,209]. For example, they can have direct effects on T cell differentiation or indirectly by changing APC trafficking or cytokine production[210].

It was demonstrated[211] that the expression of chemotactic cytokines are both cell and stimulus specific, which suggests that the recruitment of cells to a site of inflammation is dependent upon the expression of specific cytokines for both the induction and maintenance of the lesion.

Four chemokine subfamilies are known[208]: 1) The C-X-C (α chemokines which includes IL-8 (CXCL8), melanoma growth stimulator (GRO-α) and epithelial neutrophil activating peptide 78 (ENA 78) which primarily target neutrophils. 2) The C-C (β) chemokines such as RANTES (regulated upon activation normal T cell expressed and secreted), MCP-1 and MIP-1α (macrophage inflammatory protein 1α), which recruits T cells and monocytes. 3) The "C" subfamily, which includes lymphocyte-specific chemotactic peptide XCL1. 4). The CX3C subfamily with only one member, CX3CL1 (fractalkine).[179]

IL-8, a potent neutrophil chemoattractant, is the classic chemokine example. It induces adherence to vascular endothelium and extravasation of neutrophils into tissues where they become activated and degranulate causing tissue damage[179]. In addition, it is a potent inducer of monocyte/macrophage activation, which produces IL-8 in response to IL-1 and TNF[212].

MIP-1 is a LPS inducible, heparin-binding protein made up of two peptides (MIP-1α and MIP-1β). It possesses chemotactic activity for macrophages[213] and can augment the inflammatory effect of these cells in chronic inflammation[214].

MCP-1, expressed in macrophages, has similar chemotactic activity and is induced by IL-1 and LPS stimulation of peripheral mononuclear lymphocytes[215,216].

Cytokine GFs are produced by a variety of cells, including those typically involved in inflammatory processes, such as macrophages, lymphocytes, endothelial cells, platelets and fibroblasts[217]. These cytokines are powerful activators of the production of collagen and other extracellular matrix components, often in an autocrine manner[217].

Colony stimulating factors (CSF) are examples of cytokines with direct effects on cell proliferation in particular the stimulation of growth of colonies of cells from bone marrow precursors[218]. CSF-1, also referred as macrophage colony stimulating factor (M-CSF), promotes the growth of macrophages, while GM-CSF promotes the growth of both granulocytes and macrophages[218,219]. CSF-1 appears to be an important signal in inducing monocytes to mature into marcrophages[220].

TGF-β is a member of one of the most complex groups of cytokine superfamilies, consisting of various TGF-β isoforms and other family members, for example, Activin A and bone morphogenic proteins (BMP)[221]. This family of cytokines is produced primarily by chondrocytes, osteocytes, fibroblasts, platelets, monocytes and some T cells[179]. It has both stimulatory and inhibitory effects on different cell types[222].

1.2.4 Cytokines Regulation

The same cytokine may act pleiotropically, having different effects on different target cells or sometimes even on the same cell, while others can act synergistically and/or antagonistically for the same result[171]. The effect of a cytokine depends on the context in which it is working, since it is unlikely that cells in a particular inflammatory situation are exposed to only a single cytokine or only one inflammatory mediator or even one cell type[223].

The first cells to appear in a site of inflammation are neutrophils. Neutrophil numbers reach peak levels approximately 24 hours after injury[102]. Their migration is stimulated by various chemotactic factors and cytokines, including complement factors, IL-1, TNF-α, TGF-β, and chemokines such as IL-8 and MCP-1, and also by bacterial LPS[224,225]. IL-8 and IL-1 besides being a chemoattractant for neutrophils, respectively induce degranulation and activate the oxidative metabolism of those cells, causing tissue damage.[179] In addition, pro-inflammatory cytokines such as GM-CSF and TNF-α modulate NADPH oxidase activity inducing the release of large quantities of superoxide anion ($O2^{-}$)[226] in a phenomenon known as the respiratory burst. Regulation of this free radical production is critical to kill pathogens without inducing tissue injury[226].

Monocytes enter inflammatory sites where they develop into macrophages under the influence of a number of inflammatory mediators derived from other migrating cells (lymphocytes, macrophages and granulocytes) as well as from the affected tissue and endothelial cells themselves[227]. These are the cells that essentially control and regulate the wound healing process and wounds cannot heal without the participation of these cells as shown by experiments involving depletion of wound macrophages[228,229].

Monocytes are recruited following interaction with chemotactic peptides such as bacterial peptides, complement fragment C5a, leukotriene B4, fibronectin and fragments of basement membrane proteins[230]. Chemokines such as MCP-1,2 and 3, MIP-1 and 2 and RANTES also contribute to the recruitment of circulating monocytes within tissues.[227] The profile of cytokines secreted by activated and resident tissue macrophages is different[231], which allows for the modulation of most of the macrophage functions and cell surface marker expressions. Some cytokines (IL-3, GM-CSF, IFN-γ) can up-regulate the production of other cytokines by macrophages while IL-4, IL-10, IL-13 and TGF-β can inhibit that secretion[169]. TNF-α, IFN-α,

IFN-β and IFN-γ and also bacterial endotoxins, viruses, mitogens, and antigens induce the synthesis of IL-1[176,179]. In human monocytes, bacterial LPS induce approximately ten fold more mRNA and the respective proteins for IL-1β than for IL-1α.[175] IL-1 can also induce the synthesis of GM-CSF by peripheral blood lymphocytes and synergises with that cytokine in the induction of M-CSF[232]. In Infection, besides IL-1, TNF-α, IL-12 and IL-18 production is stimulated by LPS. IL-12 is generally considered the major inducer of IFN-γ production by T and NK cells[191,233]. Pro-inflammatory IFN-γ stimulates the bactericidal activity of phagocytic cells and, therefore, boosts the innate response[190]. In monocytes and macrophages besides the secretion of TNF-α, IFN-β, IL-1α and β[234-236]. IFN-γ induces the transcription of genes encoding G-CSF and M-CSF and also stimulates the release of reactive oxygen species (ROS)[193]. In addition to those stimulatory effects, IFN-γ can exert some inhibitory activity on the production of other inflammatory mediators such as IL-1, IL-2, IL-8, IL-10 and MCP-1, in human monocytes[237-240]. Bacterial endotoxins together with IL-1, TNF, PDGF, and Oncostatin M also represent physiological stimuli for the synthesis of IL-6[241]. The synthesis of IL-6 in human alveolar macrophages is however inhibited by IL-4, which prevents the production of IL-1, TNF-α and prostaglandins in response to activation of the cells by bacterial endotoxins or IFN-γ[242] Furthermore, IL-4 induces the formation of foreign body giant cells (FBGC) from human monocyte-derived macrophages *in vitro* which can in turn be reinforced by the action of GM-CSF and IL-3[243].

The expression of certain cell surface markers have been often addressed[244,245] as an index of cellular immune function and suppression and shown[246-252] to be influenced by cytokines action. Inflammatory mediators such as TNF-α[248], IL-1α[251], IL-4[249], IL-10[250] and prostaglandins[246] are known to regulate MHC-II expression. IFN-γ also regulates the expression of MHC class II genes and is the only interferon that stimulates the expression of these proteins. Due to a direct correlation with depressed MHC-II expression and defective antigen presentation[248], a monocyte population with up-regulated MHC-II expression is important for certain healing processes. IL-4 down-regulates the expression of CD14 in normal human monocytes but strongly increases the expression of CD23, another monocytic antigen[247].

IL-1 and TNF-α are also responsible for increasing the expression of adhesion molecules[252] which allow leukocytes to adhere to endothelium prior to their extravasation into tissues. IL-1 promotes the adhesion of neutrophils, monocytes, T cells and B cells by enhancing the expression of ICAM-1 and endothelial leukocyte adhesion molecule (ELAM)[179]. Lymphocytes use LFA-1 and VLA-4 to respectively bind ICAM-1 and VCAM-1.[253] In turn, neutrophils appear to use both LFA-1 and Mac-1 to attach to ICAM-1 expressing cells[153]. ICAM-1 is primarily recognised by β2 integrins while the VLA-4 molecules interact with VCAM-1[153].

Finally, the LPAM-1 integrins recognise the mucosal addressin cell molecule-1 (MAdCAM-1)[153]. In addition, the transmigration through the intercellular junction of endothelial cells appears to require the expression of platelet/endothelial cell adhesion molecule-1 (PECAM-1) another molecule belonging to the Ig superfamily[254].

Lymphocytes are, together with neutrophils, monocytes and macrophages also involved in the inflammatory process. Appropriate T_h cell development is essential for an effective adaptive immune response. It is now established that soon after microbial invasion, macrophages promptly secrete considerable amounts of IL-12, which triggers the differentiation of T cells towards a T_h1 type response[255]. Although the understanding of immune regulation is incomplete it is known that CD4+ helper T cells are capable of differentiating from an initial common state (T_h0) into 2 apparently distinct types called T_h1 and T_h2, which differ in their cytokine secretion[179,256,257]. T_h0 are responsible for the secretion of IFN-γ, IL-2, IL-3, IL-4, GM-CSF, IL-5, IL-10 and TGF-β. After differentiation IL-2, IL-3 and GM-CSF continue to be produced by both subsets while IFN-$\gamma$is only secreted by T_h1 and IL-4, IL-5, IL-10 and TGF-β by T_h2. In addition two other cytokines, LT-β and IL-6, are produced respectively by T_h1 and T_h2 cells.

The balance between T_h1 and T_h2 represents a switch, which can be used to influence the immune response in one or other direction. The commitment of T_h0 cells to become T_h1 or T_h2 is influenced by cytokines secreted by the 2 subtypes themselves and by macrophages, NK cells and mast cells.[258]

The T_h1 pathway is essentially cell mediated immunity, with the activation of macrophages, NK cells, cytotoxic T cells and a prolonged inflammatory response. A main biological activity of IL-1 is the stimulation of T_h cells, which are induced to secrete IL-2 and to express IL-2 receptors[179]. In the presence of IL-2, IL-6 induces the differentiation of mature and immature T cells into cytotoxic T cells[259]. The expression of the IL-2 receptor of monocytes is modulated by IL-5 and IL-6 and induced by IFN-γ, so that these cells become tumour-cytotoxic[260]. IFN-γ thus influences cell-mediated mechanisms of cytotoxicity modulating T cell growth and functional differentiation. It is a growth-promoting factor for T lymphocytes and improves the response of these cells to mitogens or GF. In addition, IFN-γ acts synergistically with IL-1 and IL-2[261] and appears to be required for the expression of IL-2 receptors on the cell surface of T lymphocytes[262].

The T_H2 pathway is essentially a humoral pathway, with the production of cytokines, which promote B cell growth (like IL-4, IL-6) and the production of IgG1 (IL-4), IgA (IL-5) and IgE (IL-4) in mice.[263] It also stimulates effectors, which use these antibody isotypes; eosinophils (via IL-5) and mast cells (IL-4). IL-4 plays a pivotal role within this pathway. In activated B cells, IL-4 stimulates the synthesis of IgG1 and IgE and inhibits the synthesis of IgM[264]. This

isotype switching induced by IL-4 in B cells is antagonised by IFN-γ[179]. IL-2 promotes the proliferation of activated B cells but this requires the presence of additional factors, for example, IL-10[265]. IL-6 is capable of inducing the final maturation of B cells into immunoglobulin-secreting plasma cells if the cells have been pre-activated by IL-4. The growth of B cells induced by IL-4 is however, directly inhibited by the synergistic action of IFN-γ, TNF-α and TNF-β[266]. IL-3 also inhibits the proliferation of human B cells stimulated by IL-2 by antagonising the IL-2-induced effects in B cells and by causing a slow decrease of the expression of IL-2 receptors[267]. In contrast, IFN-γ and Anti-Ig co-stimulate the proliferation of human B cells although not of murine B cells.

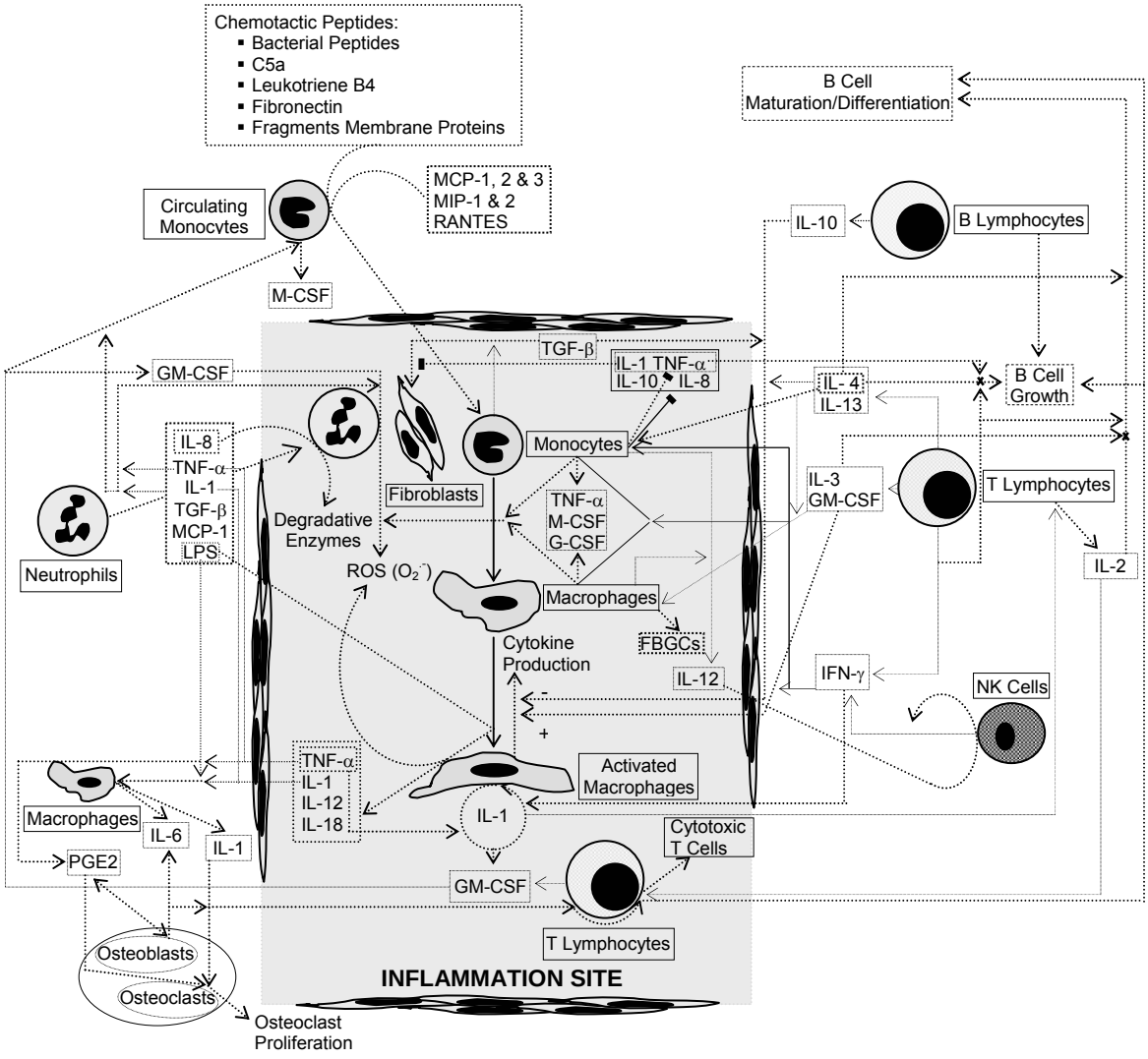

Figure 1.1. A schematic representation of some of the biological elements involved in the cytokine network.

Macrophages also control the degradation of the extracellular matrix and regulate remodelling of the wound matrix by secreting several neutral proteinases such as elastase,

collagenase and plasminogen activators.[268] Fragments of extracellular matrix and fibrin degradation products can be phagocytosed and degraded, which together with the cleavage of plasminogen into plasmin, results in the onset of important inflammatory processes involving not only activation of fibrinolysis, but also the complement, kinin and coagulation cascades. Macrophages also secrete GF such as TGF-β and PDGF, which have been shown to stimulate the growth of fibroblasts. TGF-β appears to be the major factor responsible for the formation of granulation tissue and the synthesis of proteins of the extracellular matrix[179], which have lead to it being attributed as a wound hormone. TGF-β1 and TGF-β2 inhibit IL-1 mediated proliferation of lymphocytes and at the same time decrease the secretion of inflammatory proteins such as neutral proteases. Therefore IL-1 activity antagonises the effects of TGF-β on the extracellular matrix[269]. The complexity of the wound healing process is illustrated by the observation that manipulation of the ratios of TGF-β superfamily members, particularly the ratio of TGF-β-1 relative to TGF-β-3, reduces scarring and fibrosis[270]. Re-epithelialization is mediated by chemotactic and mitogenic GF of the EGF family of GF.

The mechanism of inflammation seems rather straightforward, but how the immune system regulates the type of response to a given challenge is still unclear. Different types of challenges require very different protective mechanisms to be activated, an inadequate response can fail to protect the host against an organism or even cause damage to the host directly. Systemic actions can be critical and not only cells and chemical modulators at the site of inflammatory are involved in the process. Cells such as monocytes/macrophages and endothelial cells also contribute to bone remodelling by either contact with osteogenic cells or by the release of soluble factors namely cytokines and GF.[271]

In the skeletal system, TNF-α stimulates bone and cartilage resorption and inhibits proteoglycan and collagen synthesis under some conditions[184]. IL-1 induces the expression of a large variety of cytokines. LIF and IL-6 are two of those molecules which are known to stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage[272] but are also potent anti-apoptotic agents of osteoblasts[273]. In bone, the major sources of IL-6 are osteoblastic cells and not osteoclasts[274] however the main activity of that cytokine involves osteoclastogenesis and bone resorption[275]. Prostaglandin E_2 (PGE_2) is also directly related to IL-6 expression[276].

The production of IL-1, TNF-α and TGF-α is influenced by prostaglandins, in particular it's ability to stimulate bone resorption which is mediated by increased PGE_2 synthesis[277]. Large amounts of PGE_2 are produced in cells stimulated with IL-1[278]. In fact many of the biological activities of IL-1 are due to an increase of PGE_2 production[169]. *In vitro*, PGE_2 and glucocorticoids inhibit the synthesis of IL-1. The PGE_2-mediated inhibition of IL-1 synthesis,

like the inhibition of IL-1 synthesis caused by IFN-α and IFN-γ, is mediated by an increase of intracellular cAMP levels.

The effects of TNF-α, IL-1, IL-6 and PGE_2 are therefore interconnected; IL-6 stimulates osteoclasts formation inducing the release of IL-1[279] and mediates the stimulatory effects of TNF[280] while PGE_2 together with IL-6 activate osteoclasts in a paracrine way. Those cytokines act synergistically in the stimulation of osteoclast differentiation acting in the stromal cells or directly on osteoclasts and their precursors.

1.2.5. Foreign Body Reaction to Implanted Materials

The implantation of a biomaterial initiates a cascade of events, generally described as a foreign body reaction, which varies in time and in the inflammatory mediators involved[101,106]. The duration and intensity of the response depends on several elements including the extent of the injury caused by the implantation procedure, factors related with the host[281,282] and numerous properties of the implant such as chemical composition, surface free energy, surface charge, roughness, size and shape[106,108-112,283].

The significant properties of biomaterials have been the focus of much research, probably because they constitute the controllable variables[133,284-287] in the development of potential biomaterials. The emergence of biodegradable materials introduced more complexity to the biological response. Together with the foreign body reaction the material is degrading, which may lead to changes in shape, surface roughness, release of degradation products[287-289] and formation of particulates[290,291] therefore, from the host perspective, potentially new elements to respond to.

Histological analysis enables the degree and extent of the foreign-body reaction to be determined. Fibrous capsule formation around biomaterial implants is considered a normal response[292]. The thickness of the capsule is between 20 and 30 μm and is reported to be the ideal situation for biocompatibility[293]. Materials that induce a thin-walled capsule containing quiescent fibroblasts and a small number of macrophages were considered biocompatible[294,295]. Imai et al[296] reported that the threshold capsule thickness should not exceed 200-250μm for an implanted hydrogel. In addition, Marchant et al[297] have observed that the total collagen content was about 0.23-0.27mg/mg tissue dry weight in 21 days post-implantation of an hydrogel.

However, it must be clarified that the biocompatibility of any material includes biofunctionality[1]. Therefore, even if by inducing an inflammatory response the function for which the device was designed is not compromised and there are no sequels associated with the implantation of the device, it must be seen as biocompatible. For example, because collagenous encapsulation may impede the biofunctionality of implantable drug delivery

systems, in this particular application the objectives are a reduction of the capsular thickness and an enhancement of angiogenesis around such devices[298]. Contrarily, in the case of devices where diffusion is important such as sustained-release systems[299] and implantable bioartificial organs[300], encapsulation by a fibrotic capsule can be a considerable impediment to device function[301].

Wound healing is a very complex process that emerges after tissue injury with the aim to seal and make stable an area of damage[302]. This response involves a series of distinct stages which can overlap in terms of time[303]. Dysfunctions in any of these phases can induce disorders in the healing process, thus, the nature and severity of the injury are dependant on the normal course of the reparative process[304]. If the injury is minimal the repair process takes place in a short time, since the complete repair of the tissues might be accomplished by the regeneration of parenchymal cells only[2]. However, with more extensive wounds, the inflammation is more severe and the repair process involves scar tissue formation and possibly the loss of functions[304]. It is under this situation, where the inflammatory reaction is more complex, due to the persistent presence of the causing agent and with few mechanisms to deal with it, that the implant devices might be considered[2].

The phases of normal wound healing follow an orderly sequence of events that are characterized and regulated by the chronologic appearance of a number of different cell types[303]. Once these cells undergo activation i.e. phenotypic alterations of cellular, biochemical, and functional properties, thrombin inside the plasma clot induces platelets to degranulate releasing the contents of their alpha-granules which, in turn, lead to the expression of new cell surface antigens, increased cytotoxicity, increased production and release of cytokines[118,305]. These, together with other factors activate cells, which then mediate subsequent phases of the wound healing process.

The first cells arriving to the site of injury are neutrophils, reaching a peak after 24 hours, being removed by tissue macrophages when they are no longer needed[106]. Monocytes appear approximately 24 hours after injury reaching a maximum number 48 hours post-injury and fibroblasts migrate into the wound from day 3 onwards. Since monocytes mature into macrophages they can be considered an essential source of cytokines, which then drive repair processes. A variety of chemokines are also responsible for the spatial and temporal infiltration of leukocyte subsets and therefore control the integration of inflammatory and reparative processes during wound repair[101].

However, any type of disorder or tissue injury inevitably involves an acute phase reaction. Acute inflammation is the generic term used to label the complex process of endocrine and metabolic or neurological changes observed in an organism, either locally or systemically, a short time after injuries, infections, immunological and inflammatory reactions[306]. Thus, any type of disorder or tissue injury inevitably involves an acute phase reaction.

Many infections, especially where small wounds are the route of entry, are eliminated by the combination of complement and recruitment of phagocytes, which flow from the acute inflammatory response[117]. In fact, the acute reaction is initiated and mediated by many cytokines and by different types of cells, like PMN, fibroblasts, endothelial cells, monocytes and lymphocytes[148]. The development and control of the reaction occurs through the interaction of the numerous cascades of cytokines within the different tissues.

Locally, the acute phase is characterised by an increase in blood flow to the site of injury, enhanced vascular permeability, and the well-organized and directional influx and selective accumulation of different leukocytes from the peripheral blood at the site of injury/implantation[148,307]. In particular, the number of circulating neutrophils and monocytes increases. Neutrophils are highly destructive cells capable of mounting a rapid, non-specific phagocytic response, while monocytes mature into macrophages, which are responsible for the removal of necrotic tissue, the phagocytosis of foreign materials and the release of growth factors[306].

A short-time after implantation, the device has many proteins adsorbed onto its surface[308-310]. Some of these proteins such as IgG, fibronectin, complement C3 fragments work as opsonins[308,310]. Neutrophils and macrophages bind to the implant surface through receptors for those proteins, which results in cell attachment, activation, secretion of reactive oxygen species and the release of proteolytic enzymes[101,311]. These cells will attempt to phagocytose implants, if successful implant particulates may be a subsequent product, if the implant is too large frustrated phagocytosis will occur. Either process can release these powerful destructive species mediators stimulating further inflammation[121,307,312,313]. The presence of a device can induce a massive and excessive activation of leukocytes, sustaining an inflammatory response.

In addition to local activities, some systemic effects are typical of this type of reaction. Fever is a very common sign, but also cellular and biochemical changes occur in the liver with the synthesis of the so called acute phase proteins[117].

When an immune stimulus persists for a prolonged period of time, beyond that in which the immune system would normally have eliminated the antigen[125] a chronic inflammation process takes place.

Following neutrophils, other cell types like monocyte/macrophages and particularly lymphocytes (specific subsets of T-cells and B-cells) and plasma cells migrate to the site of injury[106,122]. These cells involve antigen-specific and more tightly regulated immune responses and once activated they also produce protective and inflammatory molecules. Another important characteristic of chronic inflammation is the development of blood vessels and production of connective tissue[2].

Once again the implant is determinant for the progress and resolution of the reaction. If the foreign body continues to resist, the host defence becomes more aggressive and a special type of inflammatory tissue is formed. Macrophages fuse forming FBGC, fibroblasts proliferate, collagen and proteoglycans are synthesised and angiogenesis take place to form the so called granulation tissue[2].

Regardless of the intensity of the inflammatory process a reparative phase, aiming to repair damaged tissues, is always present. The first step of the reparative phase involves the activation of the intrinsic part of the blood coagulation cascade. This starts when the injury takes place due to ruptures of the blood vessels which instigate the contact of plasma with tissue and basal membranes of cells and the exposure of sub-endothelial collagen to platelets[101]. The formation of a fibrin gel serves to fix plasma proteins and blood cells, which leads to haemostasis, and acts as a scaffolding matrix that can be populated subsequently by inflammatory cells such as neutrophils, monocytes, and macrophages as well as fibroblasts and endothelial cells. Thus, inadequate clot formation is associated with abnormal wound healing[2].

Fibroblasts and endothelial cells are the primary proliferating cells arriving, as the number of neutrophils decreases and the number of macrophages and fibroblasts in the wound area increases[101,106,122]. Fibroblasts replicate in response to cytokines and growth factors present in the surrounding tissues, which were released during the earlier phases of wound healing, and stored in the fibrin clot, which is invaded by these cells. Fibroblasts deposit the collagen that forms part of the substance of granulation tissue[106,314].

The formation of new blood vessels is initiated by endothelial cell migration and into the healing wound. The formation of new blood vessels within the wounded area is essential for normal fibroblast and leukocyte function, thus the process is maintained as long as required by various angiogenic factors[122].

The final phase is characterized by the gradual replacement of granulation tissue by fibrous connective tissue, a process that also requires locally acting cytokines. Collagen is synthesised and the closure of the wound and formation of the scar is accompanied by a decrease in cellularity, including the disappearance of typical myofibroblasts[2].

The presence of an implant also has a role in the disruption of the normal sequence of events of the reparative phase. Angiogenesis is restricted, due to the inability of capillaries to migrate through the device, as well as the pattern of collagen deposition being different, either would most likely result in slowing down the repair process and prolonging inflammation[2]. Furthermore, the presence of considerable amounts of granulation tissue may be responsible for an adjustment of the mechanisms of repair and their replacement by extensive scar tissue lacking specialised functions, instead of the ideally minimal fibrous encapsulation of the implant[292,315]

1.2.6 Hypersensitivity to metals

As it was mentioned before, besides helpful immune reactions, there is another type of immune response that has harmful consequences for the host normally described as hypersensitivity. So far, hypersensitivity reactions were sub-divided into four types, three mediated by antibodies and one mediated by T cells[125,149,316].

Type I hypersensitivity reactions are the most currently described because these correspond to the rapid allergic reaction. The symptoms resulting from exposure depend upon the site of contact. Mast cells and basophils have a high density of antigen specific receptors and become activated after the contact between those receptors and the allergen. This induces rapid cell degranulation releasing primary inflammatory mediators stored in the granules. The mediators initiate a sequence of events characteristic of acute inflammation. Furthermore, cell activation also induces the production of secondary mediators such as prostaglandins, leukotrienes, cytokines and enzymes[125,149].

The second class of destructive reactions - type II, is caused by specific antibody binding to cells or tissue antigens. The antibodies directly or indirectly cause cell destruction, through the recruitment of complement. Usually the target cells are foreign to the host, but they might not be, as in the case of autoimmune diseases, which mean that this type of hypersensitivity reaction is only found in blood transfusion recipients and patients with certain autoimmune diseases.

The type III hypersensitivity reaction has much in common with the type I response. It is also mediated by antigen-antibody immune complexes although with a different antibody and consequently without the involvement of mast cells. These complexes deposited at various sites triggering neutrophils to release their granule contents with consequent damage to the surrounding tissues.

Finally the type IV reaction also called delayed type hypersensitivity, which is the only class of hypersensitive reactions to be triggered by antigen-specific T cells. In this situation, the antigen is picked up by an antigen presenting cell, typically dendritic cells, processed and presented in association with MHCII molecules to T_h lymphocytes. Therefore, T cells become activated producing cytokines such as chemokines (chemoattractant for macrophages, other T cells and to a lesser extent, neutrophils) as well as TNF-β and IFN-γ. The consequences are a cellular infiltrate in which mononuclear cells tend to predominate.

One of the major problems of the currently used metallic biomaterials, is organometallic complexes, possibly being the result of the reaction of the corrosion products, which are considered to be antigens/allergens [91] and the metallic ions with the proteins of the host[317,318]. Metals accepted as sensitizers are nickel[319,320], beryllium[321], cobalt[318,322] and chromium[318,323] and occasional responses have been reported to tantalum[324], titanium[325] and

vanadium[326]. The most common metal sensitizer in humans is nickel followed by cobalt and chromium[319,320,327]. Although little is known about the mechanisms of interaction and the dynamics of the metallic products in vivo, there are many immunologic type responses reported and associated with cardiovascular[328,329], orthopaedic[319,325,326], plastic surgical[330] and dental implants[331,332]. These reactions are generally associated with the hypersensitivity type IV response[320,321,326,327], most likely mediated by wear debris products and leading to specific responses such as severe dermatitis, urticaria and/or vasculitis [320,324,327]. Furthermore, other effects such as metabolic alterations, alterations in host/invader interactions, formation of lymphocyte toxins, and initiation and/or promotion of chemical carcinogenesis may come together with the direct immune response[122,292,333,334].

1.2.7 Cytokines in Orthopaedics

The challenge in the development of new devices for orthopaedics is to insure long term stability, anchorage and function. Loosening of joint prosthesis resulting in failure is a major concern in the biomaterials field for orthopaedic applications[335,336], with revision surgery occurring at early or later stages of implantation depending on the cause of failure. A key factor is believed to be the generation of wear particles and the biological response to them in periprosthetic tissues[337]. Those reactions modulate the formation and resorption of mesenchymal tissue and eventually lead to some of the pathological findings in failed total joint replacements including membrane formation, periprosthetic osteolysis and implant loosening[338-340].

The adverse effect was suggested to depend more on the particulate nature of the material than it's chemical biocompatibility[341]. For example, both the size and volume (or number) of polyethylene particles are critical factors in macrophage activation and particles in the phagocytosable size range of 0.3-10μm appear to be the most biologically active[342]. A similar result was found for a given mass of polymethylmethacrylate (PMMA) bone cement; smaller particles (less than 20μm) resulted in more inflammation than larger particles (50-350μm)[343] and irregularly shaped particles produced a greater response than spherical particles. This is consistent with the fact that macrophages will tend to digest smaller particles[344] and form multinuclear giant cells to surround larger objects[345] although the differences in duration of their response also may have to be partly related with the material properties[345]. Large particles were shown to induce a more intense increase in the white blood-cell count and in the production of PGE_2[343] but other work[346] indicates that most of the particles in implant membranes are smaller than the resolution of the light microscope. The concentration of polyethylene particles accumulated in the tissue has been concluded by some research to be the most critical factor in the pathogenesis of osteolysis[347].

Thus, there is not yet any general understanding of the mechanisms by which particulate materials exert a harmful effect greater than that of the whole material. Hydroxyapatite (HA) products, well tolerated in bulk form, have been used widely in clinical medicine. However, porous HA blocks were reported[348] to have an unacceptably high failure rate in clinical applications. TGF-β1 concentration was found to decrease by the addition of HA particles in vitro but that variance was dependent on particle size[349]. In addition that effect was also suggested to be mediated by the increased synthesis of PGE_2[277,349].

There have been many studies on tissue at the bone/cement-material interface[339,350-352] and attention has been focused in analysing the retrieved specimen to measure the material degradation from the real environment and to perform biological studies on the tissues. In some of the studies the tissue was found to be fibrous granulation tissue[350,351] with wear particles being released into surrounding tissue. These particles can initiate chronic inflammation with a significant number of activated macrophages and FBGC aiming to eliminate the debris[353]. Therefore, implant-derived wear particles are thought to induce cytokines and prostaglandins which are the primary cause of osteolysis[354-356]. In fact, retrospective studies on failed implants suggest that periprosthetic osteolysis is mediated by activated macrophages and consequently by the released cytokines[357]. Other works[350,352,358] have revealed that several cytokines are produced, which suggests a critical role for cytokines in bone destruction and total hip arthroplasty (THA) loosening.

Demonstration of the production of bone resorptive cytokines in response to wear debris does not, however, demonstrate that bone resorption is only increased by this mechanism, since wear particles also induce production of factors that inhibit bone resorption like IL-4 and IL-10 [359]. IFN-γ for example is involved in the processes of bone growth and inhibits bone resorption probably by partial inhibition of the formation of osteoclasts. At the same time, IFN-γ synergises with LPS in the induction of NO production.

Particulate wear debris has been shown to alter the function of a variety of cell types within the periprosthetic space including macrophages, fibroblasts and osteoblasts, and either directly or indirectly osteoclasts[360-362]. Particulate debris induces monocyte/macrophage activation by multiple signalling pathways[363,364]. The interaction between particulates and cell membranes increases cytokine release without requiring phagocytosis[363]. In addition, the selective opsonisation of orthopaedic implant wear particles by human serum proteins was also shown to influence monocyte/macrophage activation[364].

Strong evidence has been shown[362,365,366] for the major role of increased recruitment of osteoclast precursors namely macrophages, and their subsequent role in wear particle-induced osteolysis, while osteoclast activation and survival appears to play minor roles. In addition, a synergistic effect of cell activation and wear particles on O_2^- production by activated macrophages and osteoclasts, suggested O_2^- involvement in mediating

osteolysis[367]. Osteoclasts are capable of producing ROS which were suggested to play a role in normal bone resorption at osteoclast-bone interface[368]. Low-levels of ROS play a role in the differentiation of pre-osteoclasts thus, if produced by macrophages or osteoclasts in response to cytokines and wear debris, can increase osteoclast formation[367,369]. Furthermore, EGF and TGF-α[370], mainly responsible for wound healing, can induce bone resorption partly due to their ability to increase the proliferation and fusion of osteoclast precursors, leading to an increase in the number of osteoclasts [371,372].

NO seems to play a role in stimulating resorption of bone by macrophages and osteoclasts[373-376]. Analysis of revision tissue has identified the presence of functional inducible NO synthase in activated macrophages and endothelial cells containing metal, polyethylene and polymethylmethacrylate (bone-cement) particles[377,378] . Nonetheless, the effect of biomaterials on macrophage production of ROS and reactive nitrogen species is largely unexplored. Those species are known to damage extracellular matrix and to increase their degradation by proteases[379,380], but they also elicit an increase in cytokine production at the implant-bone interface[381]. During inflammation, ROS have been reported[382] to activate collagenase and initiate bone resorption, another finding to support their role in osteolytic processes that cause aseptic loosening.

Macrophages respond to wear particles by releasing the pro-inflammatory cytokines IL-1, IL-6, TNF-α, as well as other bone-resorptive fibroblast-derived mediators such as PGE2 and matrix metalloproteinases[383-386]. Cemented prostheses showed higher incidence of severe osteolysis, and higher level of cytokines[387]. It has also been reported[388] that bone resorption occurred as a result of the macrophage inflammatory response to particulate polyethylene. Local bone-resorbing agents like IL-1α[389,390], IL-1β[389,391,392], TNF-α[389-391] and LPS[389,390,392] greatly induced IL-6 mRNA expression in both cell line and primary osteoblast cells.

In combination with TNF-α, IL-1 appears to be involved in the generation of lytic bone lesions[393]. IL-1 activates osteoclasts and therefore suppresses the formation of new bone.

Osteoblasts are exposed to the cytokines released in the periprosthetic space. TNF-α[394,395] and PGE2[394,396] have demonstrated negative impact on a variety of osteoblast functions such as suppressing procollagen α1 mRNA expression[394-396] and subsequent reduction of type I collagen synthesis[396]. TNF-α induces the release of IL-6[397], IL-8, MCP-1 and TGF-β. Secreted IL-6 together with PGE2 activates osteoclasts. In addition, a direct effect of wear debris on osteoblasts showed increased production of IL-6 and PGE2 and a direct influence in bone collagen mRNA expression and on the biosynthesis of bone collagen.

Lukacs et al[398] reported MCP-1 and MIP-1 production by fibroblasts in inflammatory granuloma and these chemokines were also found in membranes retrieved from total joint arthroplasty[399]. Considering that fibroblasts are a source of C-C chemokines, they can act as

chemoattractants for inflammatory cells in response to wear debris. Fibroblasts may also play an important role in osteolysis by increasing the synthesis of metalloproteinases and the secretion of certain mediators that suppress the expression of collagen.

In some cases of aseptic loosening, T lymphocytes were shown to be present, together with debris-containing macrophages, which suggest a sensitivity reaction to those particulates[376]. The recruitment of T lymphocytes to tissue interface membranes of aseptic loosened devices was confirmed by another work although those cells were not participating in hypersensitivity responses.[385] Furthermore, it was found that, in mice[400] there is a lymphocyte-independent pathway of macrophage activation in response to particulate polymethylmethacrylate. This suggests that the foreign-body response to particulate orthopaedic biomaterials is macrophage dependant and that lymphocytes are not essential to this response, although they may modulate it.

Bone resorption results in further loosening of the prosthesis, changes in stress, frictional wear, release of more wear debris and recruitment of more macrophages. Bone death and proliferation of macrophages, thus appear to be the cause for pain and loosening of prosthesis.

Because many promising materials and designs have failed in clinical use an understanding of the mechanisms involved in osteolysis is crucial to the development of new methods to prevent implant loosening. Extensive theoretical and experimental testing is mandatory before introducing new materials and implants in a clinical application. To date, many different materials have been tried in order to reduce wear and the generation of macrophage stimulating submicron sized particles, or to provide more biocompatible components. Therefore, several studies[401-404] have been carried out with potential biomaterials in order to try to understand which conditions can modulate inflammatory cell activity in response to the implanted material. By testing the cells that give rise to particular tissues rather than the tissue itself, the biological effects of biomaterials on the soft tissue can be elucidated.

Immediately following implantation, proteins adsorb onto the surface of the device therefore, the effect of the type and amount of proteins as well as the dynamics of adsorption on cytokine production has been in focus[401,405]. A hypothesis of controlling the inflammatory response of implanted devices has emerged. Natural and synthetic polymers, with variable and selective protein adsorption, have been used to coat other materials expecting to passivate within certain limits those materials[402]. The surface chemistry of a biomaterial implant can determine the degree of monocyte and macrophage adhesion and consequently the types and levels of secreted cytokines[401,402]. Therefore, the surface chemistry of the material directly and/or indirectly dictates monocyte adhesion and macrophage activation and fusion by determining the type, amount and conformation of adsorbed proteins.

Brodbeck et al[404] showed that hydrophilic and anionic surfaces promote an anti-inflammatory type of response in vitro[404] and decreased rates of monocyte/macrophage adhesion and fusion *in vivo*[404] proving that biomaterial adherent cells undergo biomaterial dependent responses, sometimes affecting the surrounding implant environment.

However, there are still some reservations in drawing conclusions, not only because the *in vitro* studies can be considered limited due to the lack independent and dependant variables or to simplistic conditions which do not represent the *in vivo* environment, but also because some results may be contradictory[171,172]. For example, polymers that supported the highest number of adherent monocytes also elicited the lowest levels of pro-inflammatory cytokines secretion[402]. Conversely, chitosan based hydrogels were found to inhibit the adhesion of macrophages, maintaining their viability without significantly affecting the production of IL-6 and TNF-α[403]. However, cytokines *in vivo* rarely, if ever, act alone.

Another concern involves the secretion profile of macrophages which is dependent on their stage of differentiation and on environmental stimuli[231]. Human macrophages obtained from various anatomical sites in the absence or presence of an inflammatory reaction also show differences in their spontaneous and stimulated release patterns[406,407]. Differentiation of monocytes in vitro in the presence of various stimuli, like bacterial antigens, lymphokines and monokines alter their subsequent secretory pattern upon stimulation with membrane-activating agents[408].

1.2.8. Immunoreactivity to Natural Origin versus Synthetic Biodegradable Systems

Currently used biodegradable materials for biomedical applications are mainly synthetic[92-96,286,409]. Natural origin biodegradable polymers such as polypeptides[410-413], polysaccharides[414-418] and bacterial polyesters[419,420] have been proposed as an alternative.

Unpredictable adverse reactions to some commonly used traditional implants have been reported during the years[82,84,97,98,421]. Nevertheless, polylactides and it's derivatives are the most commonly used synthetic biodegradable materials and they are widely accepted as biocompatible[422-425]. In the first phases of polylactide degradation in vivo, only hydrolysis takes place. The final products of the disintegration have to be removed by cells, which would normally be involved in inflammation. In fact, both clinical applications[82,84,97] and animal studies[113,114,426] have suggested that degradation products directly and indirectly affect tissue remodelling, respectively by interaction with the cells responsible for the formation of *de novo* tissue and through the induction of inflammatory cytokines released by activated macrophages. Therefore, the influence of the degradation time was addressed with long-term in vivo studies, but the results were not conclusive demonstrating acute to mild inflammation depending on the material[113,290,427]. Long-term evaluation of implanted PLLA

screws and plates[84] showed that some patients presented intermittent swelling at the site of implantation which was classified, after investigation of the nature of the tissue explanted, as a non-specific foreign body reaction to the degraded PLLA material. Furthermore, remnants of degraded PLLA were surrounded by a dense fibrous capsule and internalization of crystal-like PLLA material in the cytoplasm of various cells was also noted[84]. A comparable result was observed in another study[82] which applied poly glycolic acid (PGA) and lactide-glycolide copolymers. Only 7.9% of patients developed complications, identified as a non-bacterial inflammatory response which was typical of a non-specific foreign-body reaction. A more serious complication was detected four months after osteosynthesis of medial malleolar fractures with PGA Biofix® implants[99]. Lymphocytes were the main type of cell found in the retrieved cell suspension with a low number of mononuclear phagocytes, which suggested a lymphocyte-mediated immunological reaction against the implant.

Some studies comparing non-degradable and biodegradable materials[103,290,428,429] reported a less favourable host reaction when degradable materials were used, although the differences become less noticeable for longer times of implantation.[290] Furthermore, the expression of MHC II has been reported in response to several materials, independently of being biodegradable, such as poly(L-lactide acid)[430], hydroxyapatite-coated prostheses[431], polymethylsiloxane[432] and titanium[433].

Many uncertainties are still present but factors have been implicated in the occurrence and intensity of an inflammatory response against biodegradable implants. The difference in the rate of degradation and subsequently the difference in the kinetics of the release of the degradation products, such as monomers, oligomers and finally fragments have been considered of major importance. The issue is that the velocity of degradation might be too fast allowing the inflammation process to take over, thus compromising the role of the device[434,435]. The inflammatory cell reaction has been reported to be more intense for polymers that deteriorate rapidly[103,114,116]. However, a too slow or hardly detectable degradation can also be undesired for some applications such as the use of biodegradables to support osteosynthesis. Pistner et al[436], showed that although the polylactides presented moderate inflammation, characterised by macrophages and giant cells only during the first few weeks of implantation, some of those polymers did not present an adequate degradation rate for the proposed function.

Degradable glasses were found to stimulate an inflammatory response in soft tissue[100] which was clearly associated with its degradation rate. No chronic inflammation was observed after implantation of a slowly degrading glass, while the fastest degradation rate leads to tissue damage and necrosis. The high numbers of mast cells in fibrous tissue at an implant site was suggested to be linked with allergic reactions to the presence of glass.

The degradation process seems in turn to be influenced/controlled by other variables. It has been suggested that geometry and dimension of an implant influence biodegradation. Typically, implantation of a porous material does not induce a dense fibrous capsule[102,437]and enables a better vascular invasion of the polymer bulk[298,438,439].

Several studies[104,440-444] have highlighted other extrinsic and intrinsic factors with influence in the biodegradation of biomaterials and consequently in the tissue response. Among them it is possible to refer to pH[440] and the type of electrolytes of the degradation media [104,442], the external stress/strain applied[442], the temperature[441,443], free radicals[104,444]. It has been suggested that the results of biocompatibility studies must be aware of species differences[445,446] and the site of implantation[447,448] of the models in use.

With the emergence of natural origin materials, the research approach assumed that a combination of synthetic macromolecules with natural macromolecules might yield materials[133,449-453] whose properties would combine the advantages of both. In fact, collagen-p(Hema) hydrogels were found to be well-tolerated when subcutaneously implanted in rats[449] overcoming the problem of enzymatic resistance and inertness. Histopathological data indicated that the tissue reaction at the implant site progressed from an initial acute inflammatory response characterized by the presence of eosinophils and polymorphs to a chronic response marked by few macrophages, foreign body giant cells and fibroblasts. An artificial connective tissue matrix constructed from the association between elastin or elastin-solubilised peptides and type I + III collagens was investigated[454]. Biocompatibility studies in rabbit indicated that the material is totally integrated into the surrounding tissue after a moderate inflammatory response.

Grimandi et al[455] proposed to develop an injectable bone substitute for percutaneous orthopaedic surgery composed of methylhydroxypropylcellulose and biphasic calcium phosphate, which showed preliminary encouraging results in vivo. The inflammatory process was shown to be resolved after 15 days of subcutaneous implantation and at the same time a decrease of calcium phosphate granules and extracellular matrix formation was observed.

An hylan gel, an insoluble form of hyaluronic acid, was presented[456] as an effective alternative for soft tissue augmentation, due to its unique properties and it's capacity of not eliciting inflammatory reactions.

Natural materials have been proposed for biomedical applications mainly due to their similarities with the biological components of the host. However, there are some issues which should be considered[439,457,458]; Animal origin biomaterials may have a further problem when compared to synthetic materials. Specific immune stimulatory effects leading to humoral immune responses have been reported [439,457] and associated with contaminating proteins from the source organism of the implanted material which accentuates the need to use highly purified grades of natural origin materials. With the growing use of collagen-based

biomaterials, questions have been raised regarding the immunogenicity of this protein in humans[459]. Furthermore, most natural origin polymers are generally degraded in biological systems by hydrolysis followed by oxidation or enzymatically[442,460,461] contrarily, the majority of biodegradable synthetic polymers which are not subjected to the action of enzymes and are hydrolysed by the action of water or serum[442].

Natural origin materials are subjected to strong physiological reactions and their degradation in vivo will depend on the enzyme concentration within each living tissue. The enzyme concentration or other physiological conditions can determine the degradation rate, however, conclusions in terms of inflammation are not straightforward. Alginates for example are not subjected to enzymatic degradation when implanted in mammals, therefore presenting a limited and uncontrolled hydrolytic degradation in vivo[462]. On the other hand, chitosan is highly sensitive to enzymatic degradation in particular to lysozyme action[463,464], a neutrophilic enzyme released to the tissues after activation. Chitosan was found to be uniformly degraded in vitro by enzymatic degradation[464] but the extensive in vivo cellular response suggested that other degradation mechanisms as well as other factors were involved in that response[464,465]. However, other works with chitosan materials[466-468] revealed a mild tissue reaction with vascularisation of the implant, involving neutrophils which resolved with increasing implantation time and changed to a fibroblastic population.

Starch-based materials, other natural-origin polymers, have been shown to be degraded by α-amylase and phagocytosed by macrophages[446,469] showing an excellent tissue reaction when implanted both in rat and mice[446,470]. In works by other groups[21,471] starch-based materials implanted in rabbits and goats performed well and without adverse reactions. The host response to cross-linked high amylose starch (Contramid®) was found to be in accordance with the main phases of the inflammatory and foreign body responses to injuries caused by implanted devices[2,102,106,307]. After 4 months only a small residual scar was apparent macroscopically and it was even related with a less severe early reaction than a skin incision and closure with suture material sham[446].

Kohane et al[345,472] proposed novel lipid-protein-sugar particles to be used as drug delivery systems. It was suggested that the particles would be biocompatible due to the natural compounds[345] together with expected faster degradation rate when compared with other polymeric delivery systems[423,473]. Their residue would not be expected to remain within the tissues, inducing the formation of FBGC. In fact the evaluation of the in vivo tissue response of two tyrosine-derived polymers (poly(DTE carbonate), poly(DTE adipate) in comparison to PLLA[474] showed that the response to PLLA fluctuated as a function of the degree of degradation and that the natural origin materials did not exhibit significant inflammation. In addition, they degraded faster, probably due to enzymatic action, allowing tissue ingrowth into the implant while no comparative ingrowth of tissue was seen for PLLA.

Polyhydroxybutyrate (PHB), a polymer made by microorganisms under conditions of nitrogen deficiency, has been proposed as a biodegradable implant material on the basis of its known degradation characteristics in certain biological environments[419]. Monofilaments of PHB have been studied in vitro and in vivo up to 180 days[475] demonstrating that these materials are not biodegradable, although susceptibility to degradation may be increased by exposure to gamma radiation. Another work[476] showed that the slow degradation rate can be modified by using a filler reinforcement and by changing the initial molecular weight of the polymer. This approach allows these materials to induce a bone tissue adaptation response with no evidence of an undesirable chronic inflammatory response after implantation up to 12 months[476].

The degradation of collagen-based materials is usually controlled using chemical crosslinkers such as glutaraldehyde, formaldehyde or hexamethylene diisothiocyanate, which also involve the potential toxicity of leaching of unreacted chemical, which will contribute to increase the duration and intensity of the inflammatory response[477]. Due to the toxicity problem of the crosslinking agents, other less toxic and more biocompatible[478-480] materials are being looked for. Some of them have been found to elicit significantly less inflammation[480] than the control collagen fibres crosslinked with the currently used agents.

The subcutaneous implantation of glutaraldehyde-crosslinked dermal sheep collagen (GDSC) showed an increase in infiltration of neutrophils with a deviant morphology when comparing with hexamethylene diisothiocyanate-crosslinked dermal sheep collagen (HDSC)[481]. Furthermore, a high incidence of calcification was observed, which may explain the minor ingrowth of giant cells and fibroblasts, and the poor formation of new rat collagen. Acyl azide-crosslinked dermal sheep collagen (AaDSC) first induced an increased infiltration of macrophages, and then of giant cells, both with high lipid formation. Other authors[482] defend that collagen crosslinking preserves material integrity for a longer time, additionally decreasing tissue responses at late time intervals, probably by reducing matrix biodegradability and antigenicity. Dermal sheep collagen (DSC) materials crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide (ENDSC) induced the same mild cellular reaction as HDSC; whereas, similar to AaDSC, the degradation rate was slow and an optimal rat collagen matrix was formed.

Following implantation, blood proteins immediately adsorb to the surface of the implants. Physico-chemical properties of the implant therefore regulate that adsorption and consequently cell adhesion. Previously, implants with increased water and carboxylic group content have been shown to inhibit macrophage adhesion and multinucleation, probably because hydrophobic interactions participate in cell-matrix interactions[483,484]. Additionally, an inhibitory effect of carboxyl groups on macrophage spreading has been reported[485]. Glycosaminoglycans (GAGs) are highly negatively charged polysaccharides due to the

presence of sulphate and carboxylic groups, which may contribute to the reduced foreign body reaction found in the presence of those materials[486].

Anionic collagen membranes, negatively charged at physiological pH, presented advantages over other collagen materials also crosslinked with glutaraldehyde[487]. Besides the controlled biodegradability, the inflammatory response progressed from an intense acute response after 3 days of implantation (polymorphonuclear cells and lymphocytes) to a moderate reaction characterised by the presence of mononuclear cells with low activity after 60 days[487].

In recent years hyaluronic acid has been used for many clinical purposes and the search for new derivates continues. The esterification of hyaluronic acid with alcohols leads to the preparation of semi-synthetic insoluble polymers (Hyaff) with different physico-chemical properties allowing modulation of its biological properties[488]. In fact, fibronectin, collagen and fibrin were shown to react readily with these polymers in vitro[489] therefore modulating the cell response. In vivo tests in rats were performed[488] with several Hyaff esters which induced different tissue reactions. Cell exudates revealed a poor polymorphonuclear infiltration for all the materials but the most hydrophilic material show an almost exclusive monocyte and macrophage population around it that was responsible for it's resorption.

In general, foreign body reactions towards implants, manifested by the presence of giant cells are frequently observed[106,314,481] and if the duration and extension of the reaction does not compromise the role of the device it can be considered harmless. However, specific immune reactions have been observed with some natural origin materials. Calcium alginate dressings have beneficial effects on wound healing by providing a moist wound environment, favourable for cell regeneration[490]. The commercially available dressings may enhance wound healing through mechanisms that induce the stimulation of monocytes to produce pro-inflammatory cytokines[491]. Although this may be advantageous due to the stimulation of cellular activity at the chronic wound site and thereby enhancing the healing process[492] it can promote unresolved chronic foreign-body reaction[493]. Novel freeze-dried alginate gel dressing low in calcium ions demonstrated the improvements, showing reduced cytotoxicity and significantly reduced foreign-body reaction when compared to commercially available calcium alginate dressings[494,495].

Though the numerous collagen-based devices are reliable and effective, it has long been recognised that some subjects may develop immunity to the collagen or other components in them. Some studies demonstrated that bovine collagen implants have weak antigenic activity and that the immune responses to this implant are typically localized reactions[459,496]. However, early clinical studies with injectable collagens[497-499] verified that approximately 3% of the population develops hypersensitivity reactions. Lymphocytes were observed at the implantation site of collagenous matrices[486,500,501]. Studies with T-cell deficient rats[500] showed that T cells play a major role in the formation of giant cell and the phagocytosing activity of

macrophages and giant cells during the tissue response to HDSC. This means that the tissue reaction to biomaterials might be modulated by controlling T-cell activation in the case of unwanted or secondary burst reactions, or in the case of too-fast degradation of biomaterials. Hung et al[79] showed that SACCHACHITIN, a chitin derivative accelerates the wound healing of skin, but also induces acute local inflammatory allergic effects when its suspension was injected subcutaneously in rats. This study also reinforced the chemotactic effect of chitin on inflammatory cells suggested before[502] although concluding it as a positive effect since the onset of acute inflammation wound facilitate early angiogenesis and faster tissue formation and wound healing[79].

This introduction was structured to support the description and discussion of the work performed during the PhD. The work was sub-divided in two main areas: i) the evaluation of the biocompatibility of starch-based polymers and composites in terms of cytotoxicity and cytocompatibility, considering a potential orthopaedic application and, ii) the assessment of the immunocompatibility of starch-based polymers and composites both *in vitro* and *in vivo*.
Therefore, the initial three chapters (chapter 2 to 4) will focus on the *in vitro* tests performed both with extracts (indirect contact) and with the materials in study (direct contact) using a mouse-fibroblast cell line (L929) and a human-osteoblast cell line (SaOs-2). The intent was in an initial stage, to screen the novel starch-based biomaterials for cytotoxicity and in a subsequent phase to test, in vitro, their potential to be used in orthopaedic applications.
The remaining chapters of this thesis (chapter 5 to 7) report on the implementation of in vitro models with different types of immune system cells aiming to predict the real implantation conditions where inflammatory reaction might dictate the success of the implant. Particular emphasis was given to specific elements such as cell adhesion molecules and cytokines, which play a pivotal role in inflammation. The correlation between the *in vitro* and *in vivo* situation was also established with the implantation of starch-based materials and composites (chapter 8).

Please note that this introduction is based on the following publications:

- **A. P. Marques,** J. A. Hunt, R. L. Reis, Natural origin degradable materials: the barrier or the passage through the immune system?, in: Biodegradable Systems for Tissue Engineering and Regenerative Medicine, Ed: R. L. Reis and J. San Roman, CRC Press, Boca Raton, USA (2004), 355-375.

- **A. P. Marques,** J. A. Hunt, R. L. Reis, Mediation of the cytokine network in the implantation of orthopaedic devices, in: Biodegradable Systems for Tissue Engineering and Regenerative Medicine, Ed: R. L. Reis and J. San Roman, CRC Press, Boca Raton, USA (2004), 377-397.
- G. A. Silva, **A. P. Marques,** M. E. Gomes, O. P. Coutinho, R. L. Reis, Cytotoxicity screening of biodegradable polymeric systems, in: Biodegradable Systems for Tissue Engineering and Regenerative Medicine, Ed: R. L. Reis and J. San Roman, CRC Press, Boca Raton, USA (2004), invited chapter, 339-353.

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SECTION II

CYTOCOMPATIBILITY OF STARCH-BASED BIOMATERIALS: POLYMERS AND COMPOSITES

CHAPTER 2

THE BIOCOMPATIBILITY OF NOVEL STARCH-BASED POLYMERS AND COMPOSITES: *IN VITRO* STUDIES

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ABSTRACT

Studies with biodegradable starch-based polymers have recently demonstrated that these materials have a range of properties, which make them suitable for use in several biomedical applications, ranging from bone plates and screws to drug delivery carriers and tissue engineering scaffolds.

The aim of this study was to screen the cytotoxicity and evaluate starch-based polymers and composites as potential biomaterials. The biocompatibility of two different blends of corn-starch, starch/ethylene vinyl alcohol (SEVA-C) and starch/cellulose acetate (SCA) and their respective composites with hydroxyapatite (HA) was assessed by cytotoxicity and cell adhesion tests. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay was performed with the extracts of the materials in order to evaluate the short-term effect of the degradation products. The cell morphology of L929 mouse fibroblast cell line was also analysed after direct contact with polymers and composites for different time periods and the number of cells adhered to the surface of the polymers was determined by quantification of the cytosolic lactate dehydrogenase (LDH) activity.

Both types of starch-based polymers exhibit a cytocompatibility that might allow for their use as biomaterials. SEVA-C blends were found to be the less cytotoxic for the tested cell line, although cells adhere better to SCA surface. The cytotoxicity test also revealed that SCA and SEVA-C composites have a similar response to the one obtained for SCA polymer. Scanning electron microscopy (SEM) analysis showed that cells were much more spread on the SCA polymer and LDH measurements showed a higher number of cells on this surface.

2.1 INTRODUCTION

Biodegradable starch-based polymers have recently been proposed as having great potential for several applications in the biomedical field such as bone replacement implants[1], bone cements[2], drug delivery systems[3] and tissue engineering scaffolds[4]. The development of new processing techniques[5] and the reinforcement with various fillers results in materials with mechanical properties matching those of bone[6]. However, other conditions should be met for a material to be considered suitable for any biomedical use.

The performance of a medical device is controlled by two sets of characteristics, those which determine the ability of a device to perform the appropriate and specific function and those which determine the compatibility of the material within the body - biofunctionality and biocompatibility[7]. As such, the approach in the assessment of material biocompatibility encompasses the evaluation of the effects of physiological environments on materials and of the materials effects on the environment[8].

The evaluation of the *in vitro* cytotoxicity of a biomaterial is the initial step on a biocompatibility study, and is usually performed using immortalised cell lines[9,10] being often a qualitative analysis, based on the morphological examination of cell damage and growth when in direct[11,12] or indirect contact[13,14] with the materials.

In the present study, the biocompatibility of starch-based polymers was qualitatively and quantitatively evaluated. Cytotoxicity tests with the extract of the materials were performed in order to evaluate the presence and or release of toxic leachables and degradation products. Cell material interactions on the surface of the polymers were observed by scanning electron microscopy (SEM) and related to the materials formulations. Lactate dehydrogenase (LDH) was quantified in order to calculate the number of cells attached to the surface of the materials.

This cytosolic enzyme has been used for many years to measure the loss of cellular membrane integrity[15,16]. Indirect measurement of LDH activity, which is present in the cytoplasm of intact cells, can occur only if cells are lysed. Since LDH activity was proved to be directly proportional to the cell number[17], a new experimental procedure was developed to suite the enzyme activity measurement test for quantification of the cells adhered on materials.

2.2 MATERIALS AND METHODS

2.2.1 Tested Materials

The studied materials were: i) a 50/50 (Wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C, Novamont, Italy), ii) SEVA-C reinforced with 30% (wt) of hydroxyapatite (HA,

Plasma Biotal, UK), iii) a 50/50 (Wt %) blend of corn starch and cellulose acetate (SCA, Novamont, Italy) and iv) SCA reinforced with 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

All the materials, both the polymers and the composites were processed into dumb-bell shaped ASTM tensile samples (2x4mm cross section) by injection moulding. The samples were then cut into appropriate sizes for the different tests. The samples were sterilised by ethylene oxide (EtO) in conditions that have been described previously[2].

2.2.2 MTT Test

SEVA-C, SCA and the respective HA reinforced composites were incubated in 20 ml of culture medium (2.5 cm^2/ml)[18] for 24 hours at 37°C with constant shaking. That is an advised procedure for biomaterials extraction in order to obtain the major toxic leachables and simulate better the short-term effect of the degradation products under conditions similar to those of human body, a dynamic environment. The extract was then filtrated (0.45μm pore size) to eliminate the possible presence of solid particles of the material. Serial dilutions were made in growth medium (1:1, 1:3, ratios of extract to medium).

L929 cultured cells were resuspended in culture medium at a density of $3.3x10^4$ cells/ml and plated (200μl/well) into 96-well micrometer plates at $6.6x10^3$ cells/well. The plates were incubated, for 24h, at 37°C in a humidified atmosphere of 5% CO_2 in air. After that, the medium was replaced by the previous prepared extract dilutions, using culture medium by itself as a control. After 72h incubation the cell culture was treated with 50μl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (1mg/ml in medium 199 without phenol red, Sigma, St. Louis, USA) and incubated for further 4h at 37°C in a humidified atmosphere of 5% of CO_2 in air. At this stage the MTT was removed and 100μl/well of isopropanol (BDH, Poole, England) was added in order to dissolve the formazan crystals. The plates were placed in the incubator for 10 minutes and then in a cold room for 15 minutes before the absorbance measurements. The optical density (OD) was read on a multiwell microplate reader (EL 312e Biokinetics reader, Biotek Instruments) at 570nm.

All materials extracts were tested in 24 replicates for each extract concentration for a minimum of three separate experiments with comparable results.

2.2.3 Direct Contact Assay

In order to characterise, morphologically, the cell behaviour when in the presence of the tested materials[19], 5ml of L929 mouse fibroblasts cell suspension, in fresh culture medium

(3.3 x 10^4 cells/ml), were added to ethylene oxide sterilised materials which had been attached centrally in 6-well plates by silastic adhesive (Silastic, Dow Corning, UK). The area occupied by the test sample corresponds to 14% of the area of each well, which means that cells were cultured at a ratio of $2.3x10^3$ cells/cm^2. Dishes with and without adhesive were used as control. The plates were incubated for 1,2 and 7 days in a humidified atmosphere of 5% CO_2 at 37°C, without refreshing the culture medium. After each time period, the cells were fixed with methanol for 3 minutes, stained with 0.4% methylene blue for 10 minutes and examined by inverted microscope.

2.2.4 Preparation of cell-material surfaces for SEM observation

Cells were grown as described before for the direct contact assay. After each time period, the cells were washed with phosphate buffer saline (PBS) solution and fixed in glutaraldehyde 2.5% in 0.1M of PBS for 30 minutes at 4°C. After the fixation, the cells were washed twice, 15 minutes each, with phosphate buffer and then dehydrated in ethanol solutions 50%, 70%, 90%, and 100%, twice and during 15 minutes for each concentration. The materials with the adherent cells were kept in 100% ethanol until being subjected to critical point drying (CPD) to avoid water contamination. The samples were then gold coated by ion sputtering (BIO-RAD Microscience Division, Watford, England) and examined by SEM using a JEOL JSM-35C equipment.

2.2.5 LDH Quantification

L929 mouse fibroblasts were grown as described before in 6-well plates in contact with SEVA-C and SCA for 1,2 and 7 days without refreshing the culture medium. After each time period the plates were incubated at -70°C for approximately 30 minutes followed by thawing at 37°C for 15 minutes. The freeze and thaw cycle was repeated 3 times and then the plates were centrifuged at 250 x g for 4 minutes to eliminate any contaminant particles resulting from cell lysis. Supernatant aliquots of each well (50μl) were transferred to a new 96-well plate and the LDH quantified using an enzymatic kit (Promega, CytoTox96™, UK). The absorbance was recorded on a multiwell microplate reader (EL 312e Biokinetics reader, Biotek Instruments) at 490nm within 1 hour. At each time period a pre-determined number of cells was seeded in the same plate as the material in order to prepare a calibration curve.
The materials were assayed in triplicate for a minimum of four separate experiments with comparable results. For each material, a well without any material was used as negative control.

2.3. RESULTS

2.3.1 Cytotoxicity Assessment

The MTT test results are presented in figure 2.1. It was observed that in spite of the decrease in the percentage of viable cells with an increase in the extract concentration, none of the tested materials showed complete cytotoxicity for the L929 fibroblasts. These results are not typical for biodegradable polymers, as their degradation tends to induce a complete cytotoxic behaviour, especially when they induce a strong pH drop (as for instance for polylactides). Consequently, the obtained results are a good indication when thinking on the future clinical application of the studied polymers. For SEVA-C, it was observed a slightly higher degree of cytotoxicity for the polymer reinforced with 30% of HA, while for SCA and its composite the opposite result was observed. The SCA polymer demonstrated an increased cytotoxic effect when compared with SCA reinforced with 30% of HA. The difference between SCA and SCA reinforced with 30% of HA was not as great as for SEVA-C and SEVA-C with 30% of HA. Both the SCA polymer and composite present a value of cytotoxicity between the value of SEVA-C and composite but nearest to the one obtained for SEVA-C with 30% of HA.

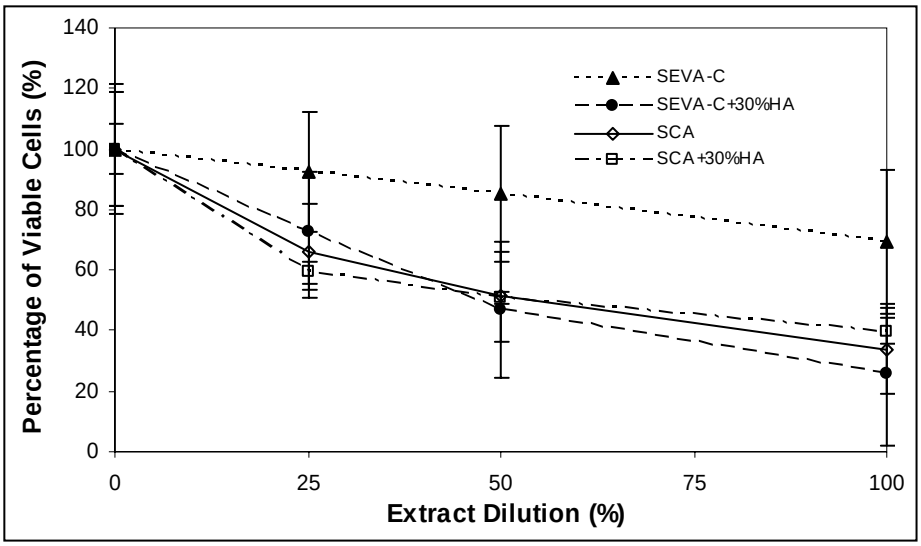

Figure 2.1. Percentage of viable cells, compared to control, after 72 hours of growth with extracts of SEVA-C, SCA and their composites.

2.3.2 Cell Morphology Evaluation

These tests were carried out for SEVA-C, SEVA-C+30%HA (Fig. 2.2). No significant morphologic changes were observed in the cells in contact with any of the tested materials for all the studied time periods. A confluent monolayer was present for the two polymers after 7 days of contact, even though SEVA-C composite presented a slightly higher degree of

cytotoxicity than the unreinforced SEVA-C. After 7 days of contact an almost confluent monolayer was observed, there was a delayed proliferation of the cells but they maintained good morphology.

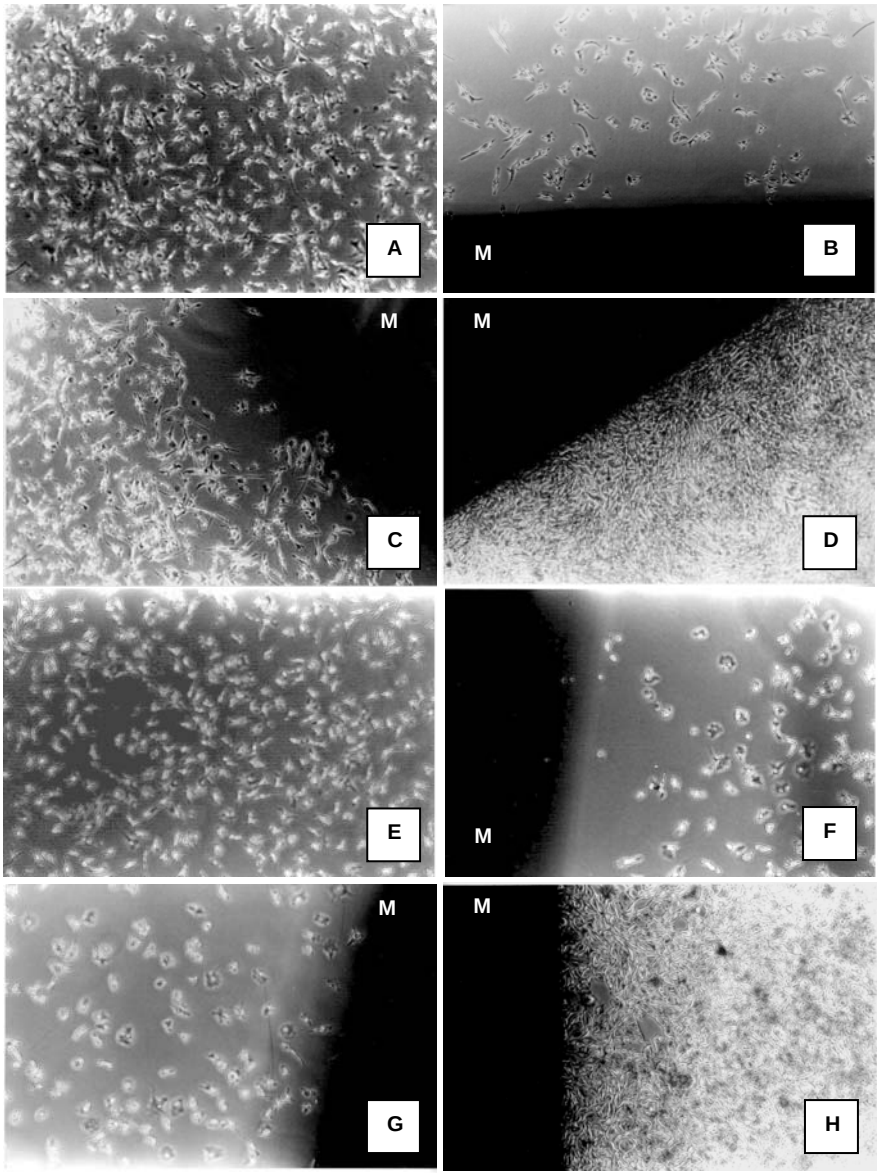

Figure 2.2. Methylene blue stained L929 fibroblasts after different time periods in contact with SEVA-C (B,C,D) polymers and reinforced with 30% of HA (F,G,H). M-material; A,E-Controls (Tissue culture polystyrene) without adhesive; B,F-One day of contact; A,E,C,G-Two days of contact; D,H-Seven days of contact (inverted microscope, original magnification x 2.5).

SEM observation complemented the previous data since it was possible to see cells adhered on the surface of the polymers. On SEVA-C and SCA, cells cover most of the surface after 7 days of growth (Fig. 2.3).

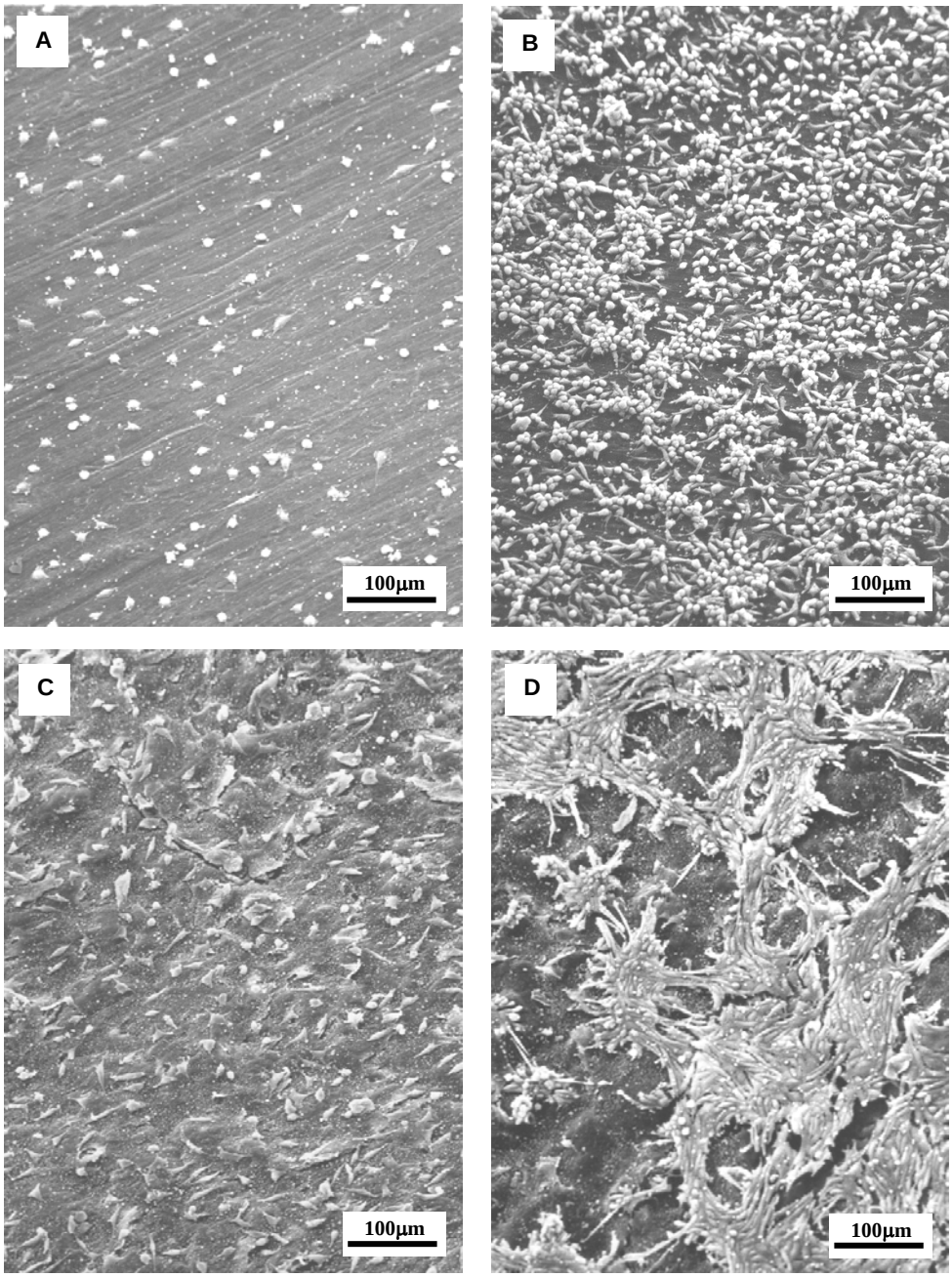

Figure 2.3. SEM micrograph showing the L929 fibroblasts adhesion to the surface of SEVA-C (A,B) and SCA (C,D) polymers. A,C-One Day of growth; B,D-Seven days of growth.

Due to the considerable difference between the materials surfaces, such as chemistry, roughness and wettability, the cell adhesion behaviour was also different. On the SCA surface the adherent cells dispersed in a more irregular way than in the SEVA-C surface. Some cells in the SEVA-C surface were not spread, they were round and sometimes connected with other cells instead of being connected to the materials surface. On the contrary, on the SCA surface the cells were well spread and connected to the materials surface by the extensive lamellipodium observed. It seems that SCA polymers present a

better surface for cells to adhere and also a relevant topography with preferential cell adhesion in specific areas.

2.3.3 LDH – Cell Adhesion and Proliferation Quantification

As it can be seen in the figure 2.4, significant cellular adhesion and proliferation was found for both starch-based polymers, SEVA-C and SCA. At the first and second days of growth, the number of adhered cells remains the same, but after seven days of growth, the number of cells on the surface of the materials is already greater than the number of cultured cells. However, the number of cells adherent to the SCA surface is higher than the number of cells on the SEVA-C.

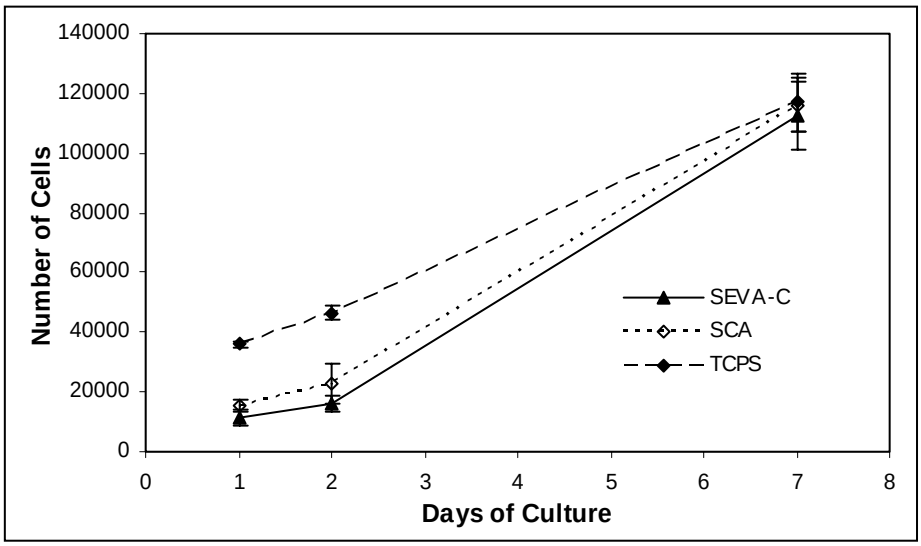

Figure 2.4. Number of cells adherent to the materials against culture time periods.

On the other hand, the total number of quantified cells in each well is not coincident with the number of seeded cells. This result is not unexpected since the tested materials present a certain degree of cytotoxicity, expected for any type of biodegradable polymer, due to the release of toxic materials (plasticizers, low molecular weight chains) during their degradation. However, they clearly pass any standard screening test needed for them to be considered as biomaterials.

2.4. DISCUSSION

The short-term effect of leachables from starch-based polymers was quantified by exposing L929 cell to the degradation products released by those materials after immersion in culture medium. The differences in cytotoxicity (Fig. 2.1), between SEVA-C polymers and composites, can be explained by some thermal degradation of the polymeric chains that

occurred during the preparation of the composites. During the processing, the polymers with reinforcement are subjected to more severe thermal and shear cycles (extrusion compounding and injection moulding) that always provoke some thermal degradation (due to viscous heat dissipation), generating low molecular weight fragments[20]. These fragments are easily leached to the solution during the extract preparation and consequently the concentration of degradation products for the same extraction period is higher for the reinforced polymers. Also it has been reported there is a preferential attack by the degradation fluids at the polymer/reinforcement interface[21]. This higher degradation rate may explain the obtained cytotoxicity data (Fig. 2.1 and 2.2). The same type of behaviour would be predicted for SCA polymers and their composites. However, the opposite was observed and this kind of behaviour could be due to the pre-purification stage (in water) performed for these materials. During this procedure the low molecular weight chains that were originated by some thermal degradation during processing, are leached out to the solution and the cellular response to the polymer and composite become more similar.

Cell adhesion and proliferation of different types of cells onto various surfaces depends on polymer surface characteristics like wettability[22,23], surface charge[24] and surface free energy and topography[25]. It has been reported there is a favoured cell attachment on moderate hydrophilic surfaces, which permits the adsorption of serum proteins with labile and reversible bonds[22]. The moderate degree of wettability of the substrates allows cells to deposit their own adhesion proteins, exchanging with the more rapidly adsorbed serum proteins. This mechanism is believed to be slower on extremely hydrophobic or hydrophilic surfaces, which is probably one reason that may contribute for cells do not adhere and proliferate so well on them[26].

The difference in cell adhesion, observed by SEM, for SEVA-C and SCA polymers may be attributed to the differences in their surface properties. The results are in agreement with the previous mentioned studies. In fact, SCA was found to be more hydrophilic than SEVA-C[27], which was also moderately hydrophilic.

However, other factors can affect cellular adhesion. Hydrophilicity, on its own is neither necessary or sufficient for cell adhesion. Chemical properties such as carboxyl[28] and hydroxyl[29,30] groups can be important in cell attachment and growth depending on the type of cell. Curtis et al[30] found that the blocking of hydroxyl groups results in lost cell adhesion but Horbett et al[31] reported that the presence of an excessive number of hydroxyl groups has an opposite effect. These studies suggest the need of an optimal density for hydroxyl groups to obtain good cell adhesion.

The topography of the materials is another parameter to consider when discussing cell adhesion. Surface-morphological studies on the roughness of biomaterials surface have been performed and showed an affinity of different types of cells for roughened surfaces[32,33].

In fact the higher water-uptake capability of SCA materials and its higher degradation rate[34] generate a rougher surface (as compared to SEVA-C) for cells to adhere. The more degraded and consequently rougher surface of SCA promoted a non-standard cellular adhesion (Fig. 2.3). The LDH quantification demonstrated that the SCA surface is the one to which cells adhere in higher numbers. These results can be pertinent considering that the cytotoxicity tests showed that SCA is the material with a slightly higher degree of cytotoxicity. However, as it was discussed, there is not only one factor to consider on its own, from the standpoint of material science, on cellular adhesion.

2.5. CONCLUSIONS

The short-term effect of the degradation products revealed that SEVA-C was the less toxic biomaterial and that its reinforcement with 30% HA induced higher percentage of cell dead due to the leaching of low molecular weight chains formed during processing (thermal degradation) and to the faster degradation rate of the composite. Despite the less promising results in terms of extract cytotoxicity, when L929 were seeded onto the materials, only a slight delaying on cell proliferation was observed in the presence of SEVA-C+30%HA but only for early culture times. The quantification of the number of cells adhered to SEVA-C and SCA did not shown a significant difference comparatively to TCPS.

Considering the overall behaviour of SEVA-C, SCA and their composites, it can be expected that their cytocompatibility will allow for their use in the future in applications such as bone replacement/fixation and/or tissue engineering scaffolding.

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CHAPTER 3

EFFECT OF STARCH-BASED BIOMATERIALS ON THE *IN VITRO* PROLIFERATION AND VIABILITY OF OSTEOBLAST-LIKE CELLS

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ABSTRACT

The cytotoxicity of starch-based polymers was investigated using different methodologies. Poly-L-lactic acid (PLLA) was used as a control for comparison purposes. Extracts of four different starch-based blends (corn starch and ethylene vinyl alcohol (SEVA-C), corn starch and cellulose acetate (SCA), corn starch and polycaprolactone (SPCL) and starch and poly-lactic acid (SPLA70) were prepared in culture medium and their toxicity was analysed. Osteoblast-like cells (SaOs-2) were incubated with the extracts and cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) test and a lactate dehydrogenase (LDH) assay. In addition DNA and total protein were quantified in order to evaluate cell proliferation. Cells were also cultured in direct contact with the polymers for 3 and 7 days and observed at light and scanning electron microscopy (SEM). LDH and DNA quantification revealed to be the most sensitive tests to assess respectively cell viability and cell proliferation after incubation with starch-based materials and PLLA. SCA was the starch blend with higher cytotoxicity index although similar to PLLA polymer. Cell adhesion tests confirmed the worst performance of the blend of starch with cellulose acetate but also showed that SPCL does not perform as well as it could be expected. All the other materials were shown to present a comparable behaviour in terms of cell adhesion showing slight differences in morphology that seem to disappear for longer culture times.

The results of this study suggest that not only the extract of the materials but also their three-dimensional form has to be biologically tested in order to analyse material-associated parameters that are not possible to consider within the degradation extract. In this study, the majority of the starch-based biomaterials presented very promising results in terms of cytotoxicity, comparable to the currently used biodegradable PLLA which might lead the biocompatibility evaluation of those novel biomaterials to other studies.

3.1 INTRODUCTION

Biocompatibility assessment comprehends several hierarchical stages each one of them aiming to evaluate the effect of different characteristics/properties of newly developed biomaterials on the biological system. The emergence of novel biomaterials, in particular biodegradables, demands an adaptation of the existing test systems in accordance to their new properties. Several variables have emerged when evaluating the biocompatibility of those materials. The possible effects of the metabolites resulting from the degradation, the local and remote interactions of cells with those products and the rate and mechanism of degradation have been the focus of some studies.[1-4]

The toxic effect of the proposed biomaterials on cells is considered one of the most important issues to be evaluated. Toxicity involves the disturbance of cellular homeostasis[5] therefore affecting cellular functions that can be very subtle or lead to a multiplicity of biochemical changes. Within cellular phenomena high importance is given to cell death, cell proliferation, cell morphology and cell adhesion, which directly correlate with toxicity *in vitro*[5-7]. Loss of viability constitutes the critical consequence generated by a toxic biomaterial. A reduced biosynthetic activity[8] as well as the release of cytoplasmic metabolites[9] or uptake of non-viable stains[10], resulting from cell membrane rupture, might be indicators of cell death. In hostile environments, anchorage-dependent cells become round, detach from the substratum and die[11]. The evaluation of cell morphology is therefore a rather simple and reliable tool to predict and identify loss of cellular viability. Another sign of toxicity is a reduced proliferation rate. Several methods[8,12] have been used to quantify cell proliferation mainly based in the quantification of total protein or DNA and in the measurement of DNA synthesis following the incorporation of radiolabelled molecules.

Considering cell adhesion, it is important to emphasize that a reduced cell adhesion might not be indicative of cell death and consequently cannot be interpreted as a toxic effect[5]. In fact, if using anchorage-dependent cells, representative of the environment that the implant will face, cell adhesion is required and its absence would be considered an indication of poor biocompatibility. This should be allied to a morphological evaluation of the cells which would allow to confirm the eventual reduced cell adhesion as a signal of toxicity.

Several biodegradable polymers have been proposed for a wide range of biomedical applications[2,3,13-18]. Some of them were considered to induce an appropriated biological response *in vitro*[15,16,18] and *in vivo*[2,16] while others provoked a negative biological effect [3,13,14,17,19]. However those materials do not exhibit comparable physical, chemical or biological properties to natural tissues therefore, the search for novel materials which resemble living systems constitutes one of the major challenges for biomaterials scientists. Natural origin materials, due to their structural similarities to components in host tissues, their possibility of

being enzymatically degraded in biological systems allowing for a better control of the degradation rate along with other properties, have been presented as potential solutions for the lack of biocompatibility of currently used devices[20-24]. Starch-based materials have revealed promising properties envisaging their use in a wide range of biomedical applications[25-28]. Therefore the aim of the present work was to evaluate the cytotoxicity of several starch-based materials (commercial environmentally applications grade) and the most currently used biodegradable material, poly-L-Lactic acid (PLLA, medical grade) in order to compare the performance of the different polymers. Cell viability and cell proliferation were the two parameters chosen to assess the cytotoxicity of the extracts of the materials and each one of the variables was quantified using two different techniques. Cells were also cultured in direct contact with the materials in study in order to compare the cell behaviour with the cytotoxicity results trying to identify potential additional negative effects of the surface of the materials.

3.2 MATERIALS AND METHODS

3.2.1 Tested Materials

The materials studied were: i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C), ii) a 50/50 (wt %) blend of corn starch and cellulose acetate (SCA), iii) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL) and iv) a 30/70 (wt %) blend of corn starch and poly-lactic acid (SPLA70).

Poly-L-Lactide (Purac biochem bv, The Netherlands), being the gold standard for biodegradables in biomedical applications, was used as a biodegradable control material and latex rubber as a positive control.

All the materials, except latex, were processed into circular samples (∅ 1cm) by injection moulding and sterilised by ethylene oxide (EtO) under the conditions previously described[25].

3.2.2 Cell Culture

A human osteosarcoma cell line SaOs-2, an immortalized cell line with an osteoblastic phenotype, was obtained from European Collection of Cell Cultures (ECCC, UK). The cells were cultured in Medium 199 without phenol red (DMGibco BRL, Life Technologies, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany), 100000 U/ml penicillin-G, 100 μg/ml streptomycin and 25μg/ml amphotericin B (Sigma Chemical Co, USA) and 20 mM Hepes (Sigma Chemical Co, USA) in a humidified atmosphere with 5% CO_2 and at 37°C.

In preparation for the MTT, Total Protein and LDH quantification tests cells were resuspended in culture medium at a density of $6.6x10^4$ cells/ml and seeded (200μl/well) in 96-well plates. For the DNA quantification, cells were resuspended in culture medium at a density of $2.4x10^5$ cells/ml and seeded (1ml/well) in 24-well plates.
All the plates were then incubated for 48h at 37°C in a humidified atmosphere of 5% CO_2 in order to establish a 90-100% confluence monolayer.

3.2.3 Extract Preparation

Materials ($3cm^2$/ml) were incubated in 10 ml of culture medium for 24 hours at 37°C with constant shaking (60rpm) in order to simulate better the short-term effect of the degradation products under conditions similar to those of human body, a dynamic environment. The extract was then filtrated (0.45μm pore size) to eliminate the possible presence of solid particles of the material and serial dilutions (25, 50 and 75%) in culture medium were prepared.

3.2.4 MTT Assay

Culture medium was replaced by the extracts of the materials (150μl/well) after cells reached the confluent monolayer and plates were incubated for 72 hours.
After incubation medium was removed, each well was treated with 50μl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (1mg/ml in medium 199 without phenol red, Sigma, St. Louis, USA) and plates incubated for further 4h at 37°C in a humidified atmosphere of 5% of CO_2. At this stage the MTT was removed and 100μl/well of isopropanol (Merck, Germany) was added in order to dissolve the formazan crystals. The plates were placed in the incubator for 15 minutes and then in a cold room for 15 minutes before the absorbance measurements. The optical density (OD) was read on a multiwell microplate reader (Molecular Devices SPECTRAMax Plus 340PC, USA) at 570nm.

3.2.5 LDH Quantification

Plates were treated with the extracts of the different materials as described for MTT test, but reserving replicates to determine total and extracellular lactate dehydrogenase (LDH). After the 72h of incubation 50μl of 10mM HEPES solution were added to each well. The solution of the wells reserved to determine extracellular LDH was transferred to new 96-well plates. The lysis of the cells adhered to the initial 96-well plates was promoted by 3 consecutive cycles of -80°C for 10 minutes and 37°C for 5 minutes and the suspension removed to another 96-well plates to quantify total LDH. Both for extracellular and total LDH quantification, 10μl of each

sample were incubated with 50μl of pyruvate (9.76mM pyruvate in 81.3mM Tris/203.3mM NaCl, pH 7.2) and the reaction was started with 125μl of NADH (0.244mM NADH in 81.3mM Tris/203.3mM NaCl, pH 7.2). Blank was read using 50μl of 81.3mM Tris/203.3mM NaCl, pH 7.2 instead of pyruvate. The LDH activity was followed through the rate of oxidation of NADH to NAD^+ for 150 seconds at 340nm (Molecular Devices SPECTRAMax Plus 340PC, USA) and the V_{max} ($OD_{340nm}x10^{-3}$/min) determined.

3.2.6 DNA Quantification

After reaching confluence, the culture medium was replaced by serial dilutions of the extract (600μl/well) of each material. Culture medium without any extract was used as control. After 72h, the extracts were removed, 200μl of trypsin-EDTA solution added to each well for 5 minutes and replaced by 1ml of phosphate buffer saline (PBS, Sigma Chemical Co, USA) solution 0.01M. The solution was homogenised with a micropipette in order to remove all the cells still adhered and transferred to new test tubes. Tubes were centrifuged for 10 minutes at 2500 rpm and 4°C, the supernatants rejected and the pellets resuspended in 5ml of Proteinase K solution previously prepared with 2,5ml NaCl 4M, 20ml EDTA 500mM, 5ml Tris 2M, pH=8.0, 25ml SDS 10% (w/v) and 525μl of Proteinase K (10mg/ml). Tubes were incubated overnight at 37°C. Following incubation 1.5ml of water plus 1.5ml of NaCl were added to each tube. These were mixed for 30 seconds and centrifuged for 3 minutes at 4000 rpm and 4°C. Supernatants were transferred to new tubes, 6ml of 70% ethanol (v/v) was add and the mixtures homogenised until DNA precipitate. Tubes were let to stabilise for 1 hour, the supernatants despised and the precipitated transferred to eppendorfs to which 200μl of 70% ethanol (v/v) and 150μl of Tris-HCl 10mM/EDTA 1mM were added.
The DNA concentration was determined reading the optical density at 260nm, using the same equipment referred to before.

3.2.7 Total Protein Quantification

As for the DNA quantification, after reaching confluence the culture medium was replaced by serial dilutions of the extract (150μl/well) of each material. Culture medium without any extract was used as control. In the end of the incubation time (72h) the extracts were removed, cells were washed with 0.1M PBS and let in 100μl of PBS 0.1M. From this point on, the BCA Protein Assay kit (Pierce Chemical Co, USA) was used. This system utilises bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. The purple coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-

soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentration.

3.2.8 Direct Contact Assay

The materials were placed in contact with cells during different time periods in order to identify morphological changes resulting from this contact and to see how cells were adhered and spread on the material.

In this assay cells were trypsinised (0.25% trypsin/EDTA solution, Sigma Chemical Co, USA) from a culture flask and 1.5 ml of cell suspension, in fresh culture medium (3,3 x 10^{-4} cells/ml) were seeded onto the materials. Three samples, per material, per time of growth, were studied and tissue culture polystyrene wells were used as control. The 24-well plates were incubated for 3 and 7 days. Culture medium was changed on the third day and after each pre-determined time of culture the cells were washed with PBS 0.1M, fixed with 2.5% glutaraldehyde (BDH, UK) solution in PBS for 30 minutes at 4°C, washed and kept in PBS at 4°C until being stained or prepared for scanning electron microscopy (SEM) observation.

The surface of the materials was therefore stained with a 0.4% methylene blue solution in water for 1 minute and examined in a stereomicroscope Zeiss KL 1500 (Zeiss, Germany). For SEM, samples were dehydrated in graded ethanol solutions (70%, 90%, and 100%) twice, 15 minutes each and let to dry overnight. Samples were gold sputter coated in a Sputter Jeol JFC 1100 and observed on a Leica Cambridge S360 SEM equipment (Leica Cambridge, UK).

3.2.9 Statistical Analysis

All materials extracts were tested in 12 (Total protein and MTT tests) and 6 (Total DNA and LDH tests) replicates for each extract concentration for a minimum of three separate experiments with comparable results.

All data was averaged and standard deviation is reported as a measure of sample deviation. The data for the neat extracts was statistically compared by a one way ANOVA analysis using a Tukey test[29]. If probability values were less than 0.05 ($p<0.05$), differences observed for the two materials were considered statistically significant.

3.3. RESULTS

MTT and LDH quantification were used to measure cell viability while cell proliferation was assessed by DNA and total protein quantification. These methodologies were applied after culturing an osteoblast-like cell line with the extracts of biodegradable polymers. Before each

test, cells were seeded in different densities and allowed to adhere overnight to confirm that each one of the parameters was linearly correlated with cell number and to define the cell seeding concentration.

3.3.1 Cell Viability

The MTT assay revealed that the extracts of all the materials in study affected the viability of osteoblast-like cells. This was expectable due to the biodegradable nature of the polymers. It was possible to observe (Fig. 3.1) that the extract of the polymer of starch with cellulose acetate induced the highest percentage of cell death (about 75%). While in the presence of the extracts of all the other materials the number of viable cells was comparable to the number of viable cells in the negative control (tissue culture polystyrene -TCPS), in the case of SCA its behaviour was closer to the positive control (latex). In fact, the percentage of cell death in the presence of the extracts of starch-based materials (except SCA) and PLLA was around 30%, which can be considered a good result for this type of polymeric biomaterials.

The statistical analysis of the results obtained for the neat extract confirmed that the effect of SCA extracts was significantly different from all the other materials. In addition, only the extract of SEVA-C was found to be significantly different from the extract of SPLA70 which suggests that the extract of SEVA-C was the less toxic (31% of cell death) and that SPLA70 was the material with second highest index of cytotoxicity (36% of cell death).

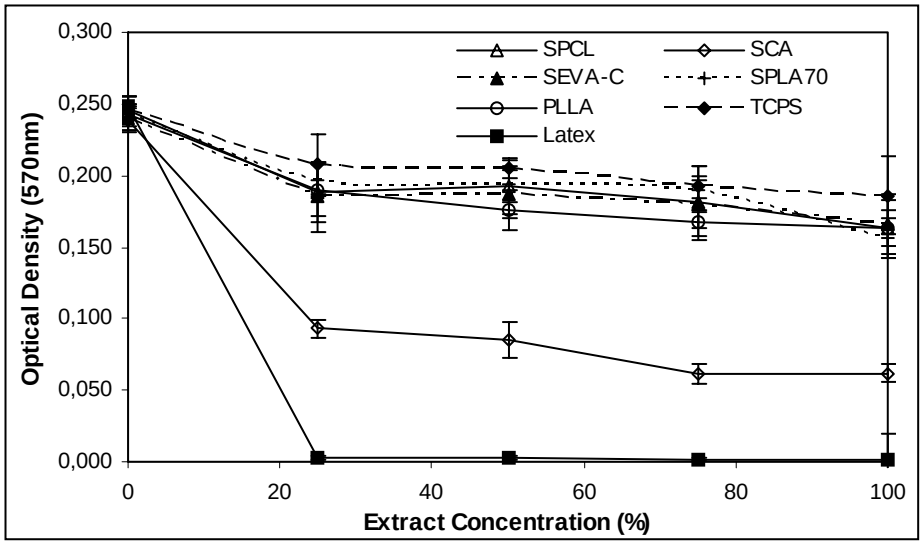

Figure 3.1. Effect of the concentrations of the extract of several starch-based polymers on cell viability when compared with controls and reference materials. The results obtained in the presence of neat extract of SCA were found to be significantly different from the results obtained in the presence of all the other materials. In addition, when comparing SEVA-C and SPLA70, their effect on cell viability was found to be significantly different.

It is known that the intracellular LDH is proportional to the number of cells[9]. This parameter was determined subtracting the extracellular LDH to the total LDH, in order to obtain the number of viable cells and compare the results with those obtained in the MTT test. In fact, although with some differences, the same tendency was observed with the LDH quantification experiment. The incubation of osteoblast-like cells with the extracts of the polymers induced a decrease in the number of viable cells (Fig 3.2). Once again, the extract of SCA induced highest percentage of cell death (about 46%). However, this was a less pronounced reduction, in comparison with the result obtained for MTT quantification.

The statistical analysis of the results however, evidenced significant differences between the materials. While the SCA effect was found to be significantly different from all the other polymers with the MTT test, the LDH quantification showed that SCA and PLLA induced a similar outcome. Furthermore, PLLA was also found to provoke significant and more cell death (about 44%) than SPCL (about 24%) and SEVA-C (about 35%). Interestingly, the toxicity of SPLA70 (about 34%) was shown to be significantly higher than the toxicity of SPCL and lower than SCA (about 46%) but not different from SEVA-C and PLLA. It is important to remind herein that SPCL and SPLA70 have both 30% of starch and 70% of PLA or PCL.

Thus, based on the LDH quantification, PLLA could be considered to be the material with higher index of cytotoxicity after SCA, and SPCL the less harmful. The toxicity index of SCA can be explained due to the release of low molecular weight chains released to the extraction medium, which are responsible for a pH drop therefore inducing cell death.

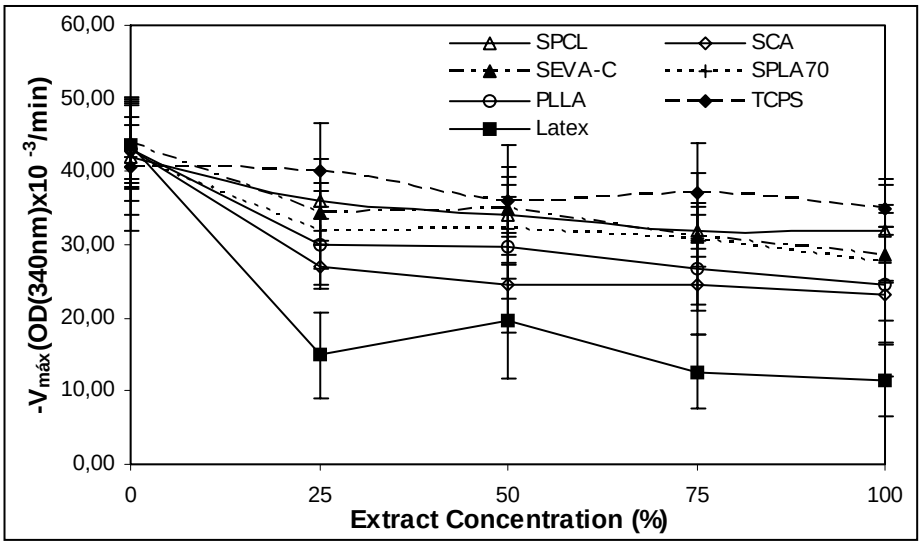

Figure 3.2. Effect of the concentrations of the extract of several starch-based polymers on the intracellular LDH activity when compared with controls and reference materials. The results obtained in the presence of neat extract of SEVA-C were found to be significantly different from the results obtained in the presence of SCA and PLLA. Furthermore, SPCL was found to induce a significant different behaviour when comparing to SCA, PLLA and SPLA70. SCA and SPLA70 were also found to be different.

3.3.2 Cell Proliferation

The proliferation of osteoblast-like cells evaluated after incubation with the extracts of the degradable materials in study showed that their degradation products affect that cellular parameter (Fig. 3.3). The quantification of DNA showed that SEVA-C and SPCL were the two polymers which had less effect on cell proliferation, respectively 26% and 28% of growth inhibition, presenting a result close to the negative control. Again, these results are quite promising for biodegradable polymers. Furthermore the statistical comparison of the results obtained with the neat extract of those two materials and each one of the other polymers showed that the reduction in cell proliferation was significantly different. The blend of starch with poly-lactic acid followed SPCL in terms of percentage of inhibition of cell proliferation (about 31%). PLLA was the material which provoked the second highest reduction in cell proliferation (about 35%) and SCA was again the material with the most negative properties inducing around 57% of inhibition of osteoblast-like cells proliferation. As mentioned before, the presence of low molecular weight chains in the SCA extract affected the cellular metabolism inducing, in some cases, cell dead and delaying proliferation of the less affected cells.

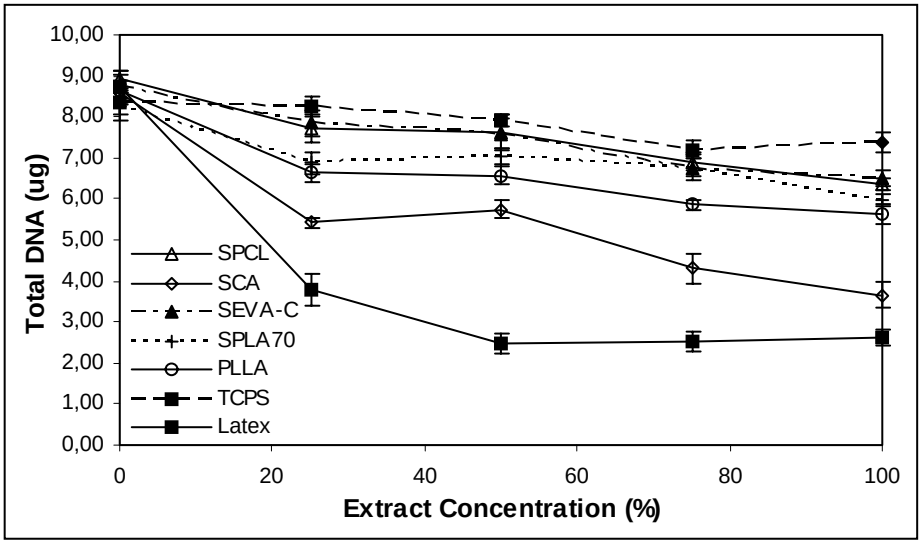

Figure 3.3. Effect of the concentrations of the extract of several starch-based polymers on the quantified total DNA when compared with controls and reference materials. Only in the presence of SEVA-C and SPCL neat extracts the effect in cell proliferation was found to be similar to negative control.

The quantification of total protein confirmed the majority of the results obtained with the DNA methodology (Fig. 3.4). Again the results obtained with the extracts of SEVA-C and SPCL revealed to be comparable to those obtained for the negative control and significantly different from all the other materials. While those two polymers induced about 43% of inhibition in cell proliferation, SCA and PLLA which results were not statistically different, provoked an inhibition closer to the positive control and of about 63%. The amount of total

protein measured after incubation with the extract of SPLA70 did not show any effect with increasing concentration of extract. In fact, the cell proliferation was affected for the 25% extract concentration, with a decrease of about 17% in cell proliferation, but did not change for higher concentrations of extract. This might be an indicator that the incorporation of starch into the poly-lactic acid positively influences cell response.

Therefore, SCA together with PLLA were shown to negatively affect the proliferation of osteoblast-like cells in higher extent while SEVA-C and SPCL presented a comparable performance to TCPS.

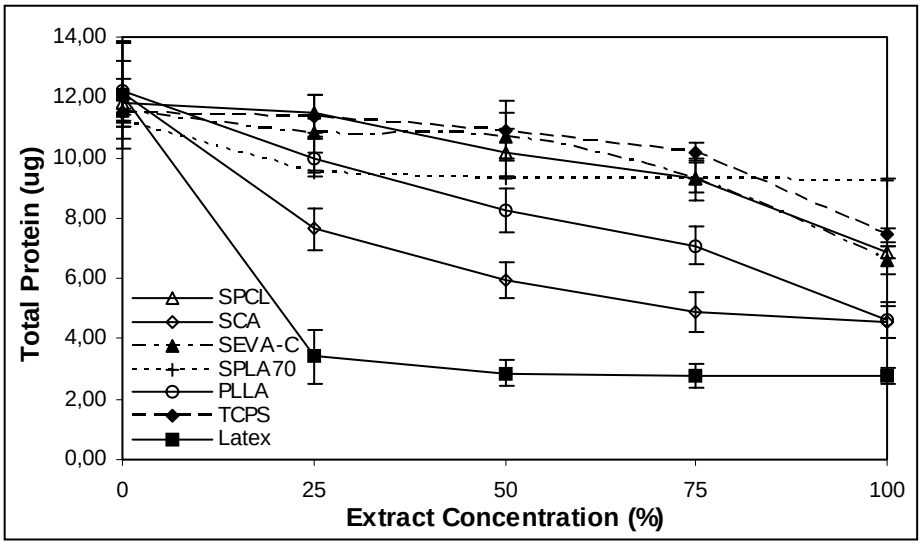

Figure 3.4. Effect of the concentrations of the extract of several starch-based polymers on the amount of total protein, as compared to controls and reference materials. The amount of protein and consequently the effect on cell proliferation was found not to be different between neat extracts of SEVA-C and SPCL, the less toxic, and SCA and PLLA the more harmful.

3.3.3 Cell Adhesion

The presence of a substrate to adhere constitutes an important variable in understanding the biocompatibility of newly developed biomaterials. Despite good cell behaviour in the presence of biomaterials extracts, it might be possible that, when in direct contact with the materials, the surface properties are not the most suitable for an optimal cell response.

Osteoblast-like cells were therefore cultured in direct contact with the polymers in study and cell morphology was analysed after 3 and 7 days. Considering the cell viability and proliferation analysis performed with the extracts of the materials, SEVA-C and SPCL were expected to be the best surfaces for cell adhesion. Figure 3.5 A and B show cells adhered to the surface of SEVA-C respectively after 3 and 7 days of culture. Cells present the typical morphology of osteoblastic cells; a polygonal shape with cytoplasm extensions looking for adhesion points on the surface of the materials. SEVA-C appears to present appropriate physico-chemical properties for SaOs-2 to adhere and proliferate since the surface of the

sample after 7 days of culture was almost fully covered. However, and contrarily to what was expected, cells adherent to SPCL did not show the characteristic morphology of osteoblast-like cells (Fig. 3.6 C and D). Although spread, cells did not seem to be strongly adhered which may prevent an adequate long-term cell response (Fig. 3.5A). In addition, the proliferation rate of these cells did not seem to be comparable to cells adhered to SEVA-C since SPCL surface area occupied by cells, after 7 days of culture, was less significant (Fig. 3.6D).

Like for SPCL, the extract of SPLA70 did not significantly affect the behaviour of SaOs-2 but its adhesion performance on the surface of that polymer was not as good as it could be expected in particular for earlier times of culture (Fig. 3.6E). Some of the adherent cells presented the typical morphology of osteoblast-like cells but there were a great number of cells that seemed flatten and not so well adherent the surface (Fig. 3.5B). Despite this, after 7 days of culture an almost confluent layer of cells was covering the surface of SPLA70 (Fig. 3.6F) showing that cell proliferation is not affected by the surface of this polymer.

Comparing SPLA70 with PLLA, the extract of PLLA showed a more damaging effect on cell viability and proliferation. However, the surface of the material was found to induce good adhesion behaviour (Fig. 3.6 G and H). Cells presented a morphology representative of an ideal adjustment to the surface with strong adhesion. Cell proliferation, like for SPLA70, did not seem to be affected also resulting in an almost fully covered surface after 7 days of culture (Fig. 3.6H).

Again, the worst results for SCA extracts were confirmed by the adhesion tests (Fig. 3.6 I and J). Cells on the surface of starch with cellulose acetate presented a round morphology and did not proliferate for longer culture times.

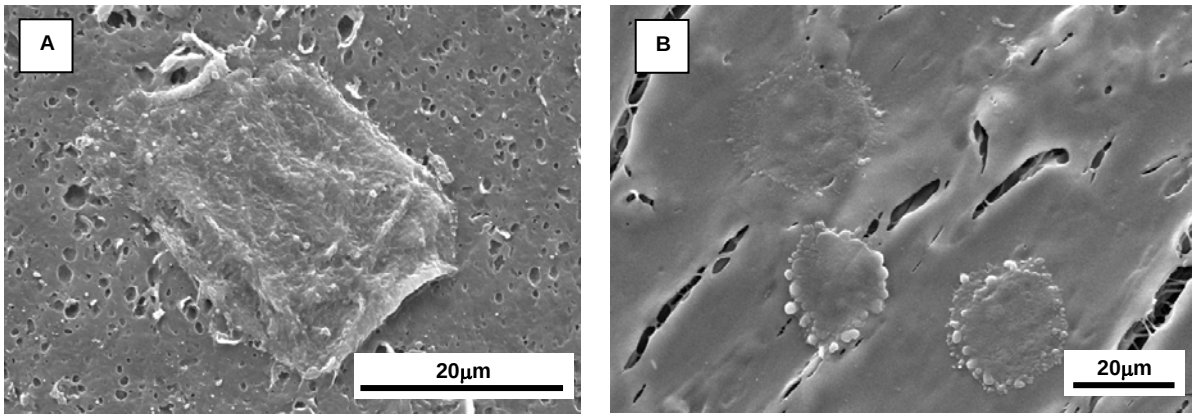

Figure 3.5. Scanning electron micrograph showing SaOs-2 cultured on the surface of SPCL for 3 days (A) and SPLA70 for 1 day (B). Bar represents 20μm.

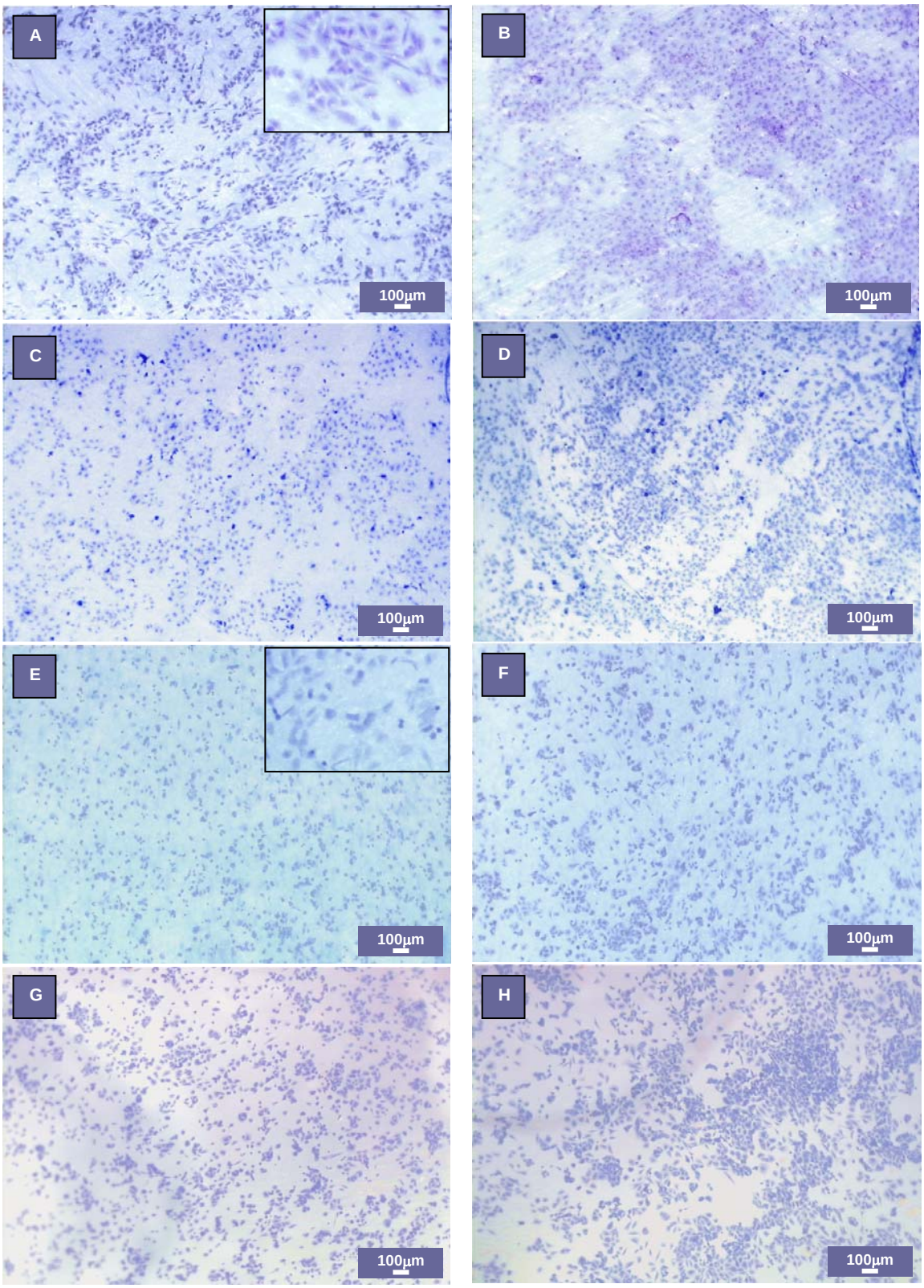

Figure 3.6. Optical micrographs showing SaOs-2 cultured on the surface of biodegradable polymers for 3 and 7 days and stained with methylene blue. A, B - SEVA-C; C, D - SPCL; E, F - SPLA; G, H - PLLA; I, J – SCA. A, C, E, G, I – 3 days of culture; B, D, F, H, J – 7 days of culture. Small squares on the upper corner represent an area of the micrograph at higher magnification. Bar represents 100µm.

3.4. DISCUSSION

The present study represents a multi-endpoint approach which provides information about different cellular functions. The aim was to use four alternative methods determine the cytotoxicity of the degradation products of biodegradable biomaterials at different levels. As typically toxic substances do not act at one specific level but affect several cellular functions[8], we determined how toxic leachables acted at cellular and sub-cellular levels the measuring the activity of mitochondrial and cytoplasmic enzymes and quantifying DNA and total protein content.

Some discussion[23,30-32] arises when comparing different methodologies to determine cell cytotoxicity, but statistically significant correlation between assay techniques were also reported[33,34]. Some authors[32,34] defend that the measurement of an intracellular parameter such as DNA content may be a more sensitive tool for the estimation of the cytotoxic potential of a test material. Furthermore, MacNair et al[31] demonstrated that LDH assay is inferior in terms of sensitivity since it represents a terminal event while the measurement of total cell protein content was presented as a more sensitive index of cytotoxicity.

In this work, however, when comparing the results obtained for MTT and LDH we could suggest that intracellular LDH is a more sensitive index of toxicity. In the LDH quantification assay higher levels of than originally thought (after the MTT test) of statistically different toxicity levels were found for the majority of the materials. As some of these materials were considered, after the MTT test, to have a similar toxic behaviour these results are clearly indicative of the higher sensitivity of the LDH technique.

Analysing the results of DNA and total protein quantification our findings are in accordance with the literature. The DNA measurement proved to be more sensitive than the determination of total protein.

Based in the obtained results, we may also speculate that proliferating cells may be more sensitive than the resting cells to a toxic challenge therefore the cytotoxicity trend be different for some materials when comparing the methodologies used to evaluate cell viability and cell proliferation.

A great challenge in the development of novel biomaterials is to support the interpretation of the cytotoxicity results in the characteristics of the materials. In particular, degradable polymers display variable behaviour in biological systems, depending on various properties such as molecular weight[1], hydrophobicity[35], distribution of charge[22], residual monomer[36] and pH of the degradation products[3]. Therefore, these factors, combined with the degradation kinetics, are important in determining the toxicity of potential biomaterials. The pH and osmolarity of polymer extracts have been suggested to be related to the toxicity of polymers[4,37] and dependent on the amounts of solubilised monomers and oligomers[38]. In

fact, pH influences cell behaviour and viability and acidic ph lower than the physical pH of the cells can cause a toxic response[33,39] and osmolarity is a factor that can exert an influence on proliferation, morphology and cell activity[36].

From the toxicity data in this work it appears that the material with higher index of cytotoxicity is SCA. This is the material with higher capability to uptake water therefore with higher predisposition for degradation. I addition SCA is a non-miscible blend which affects the kinetics of degradation releasing higher amounts of low molecular weight chains to the extraction medium at early stages of immersion. In fact, the extraction medium (culture medium with phenol red) showed a slight change of colour indicative of acidification; thus it is possible that the low molecular weight chains released to the medium are responsible for the pH decrease and consequently for the cytotoxicity.

The pH of the extraction medium does not seem to be responsible for the degree of toxicity observed for the other materials. Although not as obvious as for SCA, there are some differences between the materials, in terms of cytotoxicity, that might be attributed to the degradation products of the polymers. In fact, Ignatius et al[12] reported studies in buffer solutions where PLLA toxicity was attributed to the degradation products themselves. In another work[4] the low pH of the water incubated PLA specimens was attributed to their degradation and the resulting concentration of lactic acid in the exposure medium. It can be speculated that since the pH of the extraction medium does not change, the toxicity presented by the PLLA extracts, mainly affecting cell proliferation is due to the interaction of the cells with the products of degradation. Previous works with starch-based biomaterials[40-42], in particular with SEVA-C and SCA extracts incubated with other type of cells, have shown promising results. Thus the cytotoxicity of SCA can be attributed to the high amount of low molecular weight chains and processing additives, which can be removed by an additional processing stage.[42]

Cytotoxicity tests with extracts are usually defined as indirect contact tests. In our groups we defend that these should be complemented with direct contact tests since materials may display differences in cytotoxicity depending on cell-material contact arrangements. Cell-material contact can in a way reduce the sensitivity of an *in vitro* system but also influence cell viability, probably due to chemical interactions[18,43]. The shape[44] and surface texture[43,45] of an implant are other important factors, determining the tissue response although a conclusive mechanism is not yet established.

Cell adhesion experiments performed in this work demonstrate that besides the extracts of the materials the three-dimensional forms of the polymers have to be tested as the results of cell behaviour may drastically change. This was the case of the blend of starch with polycaprolactone (SPCL) which did not present significant toxicity but when in direct contact with the materials showed reduced cell adhesion and delayed proliferation rate. Contrarily,

SCA confirmed to be the less suitable surface for cell adhesion as was expected by the cytotoxicity test. However, other studies[46,47] with SPCL and SCA showed that these two materials processed under different conditions and shapes aiming for example tissue engineering purposes, support cell adhesion.

The different percentages of starch and the miscibility and the starch-based blends might also have some influence in the biological performance of those biomaterials. SEVA and SCA, both with 50% of starch could be expected to induce a similar behaviour however, SCA is a non-miscible blend which can contribute to a completely different surface in terms of starch and synthetic component exposure and consequently cell adhesion. In addition the two starch blends with 30% of starch SPCL and SPLA70 also presented very distinct cell adhesion results. This might indicate that in this case the synthetic component rules cell adhesion and proliferation and we can speculate that increasing the percentage of starch in the blend with polycaprolactone would improve those actions.

Thus specific surface properties have pivotal role on cell adhesion behaviour. Studies with pure PCL showed that it has an hydrophilic surface and osteoblast-like cells appear to prefer more hydrophobic surfaces[35]. Contrarily, Yang et al[18] reported less adhesion and differentiation of bone-marrow stromal cells onto hydrophobic surfaces due to less adsorption of fibronectin. The starch-based blend SPCL is more hydrophobic than the other materials which are in accordance with Yang et al[18]. However, other authors[48] suggest that some chemical groups have more significant role in cell adhesion than the general surface properties. For example, the oxygen content of SEVA-C, lower than SPCL and SCA[49], seems to be the most suitable for the adhesion of SaOs-2 under the studied conditions.

3.5 CONCLUSIONS

The data generated by this battery of assays allow for a response on the cytotoxic potential of materials or devices with a higher grade of certainty. In addition it also provides the guaranty that if the leachables from the materials interfere with one test system the results are not misinterpreted.

It was also possible to prove that not only the extract of the materials but also their three-dimensional form has to be biologically tested in order to analyse material-associated parameters that are not possible to consider within the degradation extract.

Therefore, both direct and indirect tests allowed to determine that SCA induced significant cytotoxicity and did not present the ideal surface properties for osteoblast-like cells adhesion and proliferation. Contrarily, SPCL extract was not deleterious for cells but did not support their proliferation. Comparatively to the gold standard biodegradable biomaterial, SEVA-C and SPCL showed a better behaviour than PLLA in terms of cytotoxicity. The adhesion and proliferation of osteoblast-like cells on SEVA-C and SPLA70 was however, comparable to

PLLA which indicates the good potential of the majority of the starch-based biomaterials tested for bone related applications.

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CHAPTER 4

HYDROXYAPATITE REINFORCEMENT OF DIFFERENT STARCH-BASED POLYMERS AFFECTS OSTEOBLAST-LIKE CELLS ADHESION/SPREADING AND PROLIFERATION

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ABSTRACT

The aim of this study was to determine which, from a range of the starch-based biomaterials, would be more suitable to be used in orthopaedic applications. This included blends of corn starch and ethylene vinyl alcohol (SEVA-C), corn starch and cellulose acetate (SCA), corn starch and polycaprolactone (SPCL) and its composites with increasing percentages of hydroxyapatite (HA). The polymers and composites were cultured in direct contact with osteoblast-like cells (SaOs-2) and the effect of the incorporation and of increasing percentages of the ceramic in osteoblast adhesion/proliferation has been assessed. In the evaluation of cell adhesion and proliferation rate, two variables were considered; cells adhered to the bottom of the tissue culture polystyrene (TCPS) wells and cells adhered to the surface of the materials, in order to distinguish respectively: i) the effect of possible degradation products released from the materials to the culture medium and ii) of the surface properties on the osteoblast-like cells. In addition, the morphology of cells adherent to the surface of the starch-based polymers was analysed and correlated with their topography and with other chemical properties previously evaluated.

The proliferation rate was found to differ from blend to blend as well as with the time of culture and with the presence of HA depending on the material. SEVA-C and respective composites systematically presented the higher number of cells comparatively to the other two blends. SPCL composites were found to be less suitable for cell proliferation. The amount of cells quantified after 7 days of culture, both on the surface and on the wells showed a delay in the proliferation of the cells on SPCL composites surfaces as compared to other materials and to TCPS. SCA composites however, did support cell adhesion but also induce a slight level of toxicity which results in delayed proliferation on the cells adhered to the wells.

Cell morphology on the surface of the materials was also, in almost every case, found to be appropriate. In fact, cells were well adhered and spread on the majority of the surfaces. Thus, starch-based biomaterials can be seen as good substrates for osteoblast-adhesion and proliferation which demonstrates their potential for their use in orthopaedic applications and as bone tissue engineering scaffolds.

4.1. INTRODUCTION

The evaluation of the biocompatibility of newly developed biomaterials involves numerous steps aiming to assess its safety and suitability for a proposed application. Following the early screening stage where cytotoxicity is evaluated, other concerns, directly correlated with the future application of the materials, arise. Studies start to be performed using *in vitro* culture of cells that will face the implant[1]. For example, the evaluation of biomaterials proposed for orthopaedic applications has been performed using osteoblast-like cells[2-4] and/or primary cultures of osteoblasts[2,5-7]. These are cultured in direct contact with the materials to be tested providing a rapid, sensitive and cost-effective *in vitro* evaluation, relevant to the function of the device. One of the most important parameters to evaluate is cell adhesion. While for some applications, such as hemocompatibility[8], a reduced cell adhesion is desired, for others, such as orthopaedics[7,9,10], enhanced cell adhesion and proliferation is required. Following adhesion, cells can experience activation which might be evidenced through a variety of processes including spreading, migration, proliferation and biosynthetic activity. Although the precise mechanisms of integrin-related events have not yet been fully elucidated, those processes have been correlated with changes in cell survival, cell proliferation and cellular differentiation.[11-13] Cell spreading involves complex cytoskeleton reorganisation and is an essential function of cell that had become adherent to a surface. Proliferation follows cell spreading and it is central for materials designed to be integrated into host tissues. Osseointegration for example is critical in orthopaedic applications.[14]

Cell adhesion and consequent states depend not only on the cell type[15,16] but also on the physical and chemical properties of the material surface[13,17,18]. Firstly these properties control the layer of proteins primarily adsorbed to the material which interact with the integrins, cell-membrane proteins that determine the adhesion and migration behaviour as well as cell morphology[19,20]. Although the protein layer adsorbed to the surface of the materials is known to mediate that cell-material interaction, protein adsorption appears neither to be related to a specific site of the substrate nor to induce specific orientation of the ligand. Proteins regulate early events, however they probably also initiate signalling cascades which regulate long-term events such as protein production.[12]

Previous works[18,21-23] have demonstrated that surface topography and surface chemistry play important roles in cell orientation. Therefore, not only cell adhesion, proliferation and differentiation, but also cell morphology, give information about the appropriateness of newly developed biomaterials for a specific application and can be modulated by controlling the surface of the materials.

In the present study, starch-based blends with different synthetic components previously proposed to be used in a wide range of biomedical applications[24-27], were reinforced with increasing percentages of hydroxyapatite (HA) in order to evaluate the effect of the presence and amount of the ceramic in the behavior of osteoblast-like cells in terms of cell adhesion/morphology and proliferation. Hydroxyapatite is a bioactive material known to promote the differentiation of osteoblastic cells *in vitro*[28-30]. Moreover, it was suggested[31] that the proteins adsorbed to the surface of HA induced a specific spreading behavior therefore affecting subsequent proliferation and differentiation.

4.2. MATERIALS AND METHODS

4.2.1 Materials

The materials studied were: i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C), ii) SEVA-C reinforced with 10%, 20% and 30% (wt) of hydroxyapatite (HA, Plasma Biotal, UK), iii) a 50/50 (wt %) blend of corn starch and cellulose acetate (SCA), iv) SCA reinforced with 10%, 20% and 30% (wt) of hydroxyapatite, v) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL) and vi) SPCL reinforced with 10%, 20% and 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

All the materials were processed into circular samples (∅ 1cm) by injection moulding and sterilised by ethylene oxide under the conditions previously described[24].

4.2.2 Cell Culture

A human osteosarcoma cell line SaOs-2, an immortalized cell line with an osteoblastic phenotype, was obtained from European Collection of Cell Cultures (ECCC, UK). The cells were cultured in DMEM (Gibco BRL, Life Technologies, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany), 100000 U/ml penicillin-G, 100 μg/ml streptomycin and 25μg/ml amphotericin B (Sigma Chemical Co, USA) and 20 mM Hepes (Sigma Chemical Co, USA) in a humidified atmosphere with 5% CO_2 and at 37°C.

Cells were trypsinised (0.25% trypsin/EDTA solution, Sigma Chemical Co, USA) from a culture flask and 1.5 ml of cell suspension, in fresh culture medium (3,3 x 10^{-4} cells/ml) were seeded onto the materials. Three samples per material per time of growth were studied and tissue culture polystyrene (TCPS) wells were used as control. The 24-well plates were incubated for 1, 3 and 7 days. Culture medium was not changed until the end of the experiment.

4.2.3 Microscopy Analysis

After each time of culture the cells were washed with a 0.1M phosphate buffered saline (PBS, Sigma Chemical Co, USA) solution fixed with 2.5% glutaraldehyde (BDH, UK) solution in PBS for 30 minutes at 4°C, washed and kept in PBS at 4°C until being stained or prepared for scanning electron microscopy (SEM) observation.

The surface of the materials was therefore stained with a 0.4% methylene blue solution in water for 1 minute and examined in a stereomicroscope Zeiss KL 1500 (Zeiss, Germany). For SEM, samples were dehydrated in graded ethanol solutions (70%, 90%, and 100%) twice, 15 minutes each and let to dry overnight. Samples were gold sputter coated in a Sputter Jeol JFC 1100 and observed on a Leica Cambridge S360 (Leica Cambridge, UK).

4.2.4 Total Protein Quantification

In the end of the incubation time the culture medium was removed, cells were washed with 0.1M PBS. Materials were transferred to new 24-well plates and 100 l and 500 l of 0.1M PBS were add to each well, respectively of the initial and new culture plates. From this point on, the BCA Protein Assay kit (Pierce Chemical Co, USA) was used. This system utilises bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. The purple coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentration. At the end of the assay, 100 l of each sample from TCPS wells and materials were transferred to 96-well plates where a standard curve was prepared and the absorbance read in a multi-well plate reader (SpectraMax 340PC).

4.2.5 Statistical Analysis

The total protein was quantified in four separate experiments, each one carried out with four replicates for each material.

All data was averaged and standard deviation is reported as a measure of sample deviation. The data for the neat extracts was statistically compared by a one way ANOVA analysis using a Tukey test[32]. If probability values were less than 0.05 ($p<0.05$), differences observed for the two materials were considered statistically significant.

4.3. RESULTS

4.3.1 Cell adhesion/proliferation quantification

Previously to the establishment of the protocol, serial concentrations of cells were cultured in order to prove its proportionality with the total protein amount and also to define the initial amount of seeded cells.

The results obtained in the total protein quantification assay were presented as the amount of protein measured from cells adhered to the materials (Fig. 4.1 A, C, E) and from cells adhered to the bottom of the TCPS wells used (Fig. 4.1 B, D, F). The aim was to distinguish the effects of the surface of the polymers studied and of possible toxic degradation products that would affect not only cells on the surface but also the cells adhered to the well. In the majority of the cases the obtained results were quite good and not typical for other types of biodegradable polymers.

4.3.1.1. SEVA-C and Composites

It was observed that osteoblast-like cells have a preference for the polymer and composites with a matrix of starch and ethylene vinyl alcohol (Fig. 4.1A). After one day of culture the amount of cells adhered to those materials was higher than the number of cells present in the control TCPS. In fact this difference was found to be statistically different. However, after 3 days the proliferation rate of cells in the control material allowed to reach numbers comparable to the ones observed for cells adhered to SEVA-C and composites. An exception was observed for SEVA-C+20%HA, which seemed to delay cell proliferation in such an extent that the amount of total protein after 3 days on that materials was statistically lower than on the TCPS. After 7 days of culture and as expected, the number of cells on the surface of SEVA-C and composites was found to be statistically lower than on the control. From day 3 to day 7 however, cells on those starch-based biomaterials proliferate at a considerable rate, and although SEVA-C +20% of HA seemed to be the less appropriated for cell growth, it was not found to induce a statistically different result from SEVA-C or the other composites with 10% and 30% of HA. Therefore, the reinforcement with HA did not seem to have a significant direct effect in the adhesion/proliferation of osteoblast-like cells on the surface of starch-ethylene vinyl alcohol blend, for these times of culture.

Considering the effect of the presence of SEVA-C and its composites in the metabolism of cells adherent to the bottom of the wells, there were no statistically significant differences between these materials for any of the times of culture (Fig. 4.1B). The amount of total protein was lower than the one measured for cells on the surface of those materials except for SEVA-C+10%HA and SEVA-C+20%HA which were comparable. This might be explained by a stronger effect of the surface properties of these materials in contrast with the effect of possible degradation products. For 1 and 3 days the number of adhered cells in the wells in the presence of SEVA-C and composites was found to be statistically lower when comparing to the results obtained for SCA+20%HA. Therefore, higher adhesion to the bottom of the well, comparatively to SEVA-C and composites, was observed for other starch-based materials; at the same time a higher number of cells was observed on the surface of SEVA-C and its composites. In addition, after 7 days the number of cells in the wells the presence of SEVA-C composites is higher than in the presence of SCA composites, statistically significant comparatively with SCA+30%HA. We might speculate that at early culture times osteoblast-like cells consider the surface of SEVA-C and its composites “friendly” enough to adhere/proliferate instead of migrating to the TCPS and a possible equilibrium starts to be established for longer culture times.

4.3.1.2. SPCL and Composites

The results obtained for SPCL and its composites were considerably different to what was observed for the blend of starch-ethylene vinyl alcohol. The number of cells quantified on the surface of SPCL and respective composites was statistically lower comparatively with SEVA-C and its composites at day 1. Interestingly, for this same time of culture the amount of total protein obtained from cells adhered to SPCL and its composites was comparable to the value obtained for control (TCPS) (Fig. 4.1C). At day 3 however, the proliferation rate in the TCPS had prevailed inclusively being statistically higher than on the surface of SPCL and SPCL+20%HA. Furthermore the number of cells on the surface of SPCL was also found to be statistically lower comparatively to SEVA-C and SEVA-C+10%HA for the same time of culture. As observed for the materials of starch-ethylene vinyl alcohol, after 7 days of culture the number of cells quantified for the TCPS was statistically higher than for SPCL and composites.

Contrarily to what was observed for the starch-ethylene vinyl alcohol blend, the reinforcement of SPCL had a quite strong effect on osteoblast-like cell behaviour in particular for longer times of culture. At day 7 SPCL composites were no longer suitable for cell proliferation presenting a number of adherent cells statistically lower than the unreinforced polymer (SPCL) and SEVA-C and its composites with 10% and 30%HA.

The measurement performed in the wells where osteoblast-like cells were cultured with the blend of starch-polycaprolactone, showed completely distinct results. Comparing the values obtained for the different materials of the two blends at each time of culture, only in the presence of SEVA-C the cell number was found statistically lower than in the presence of SPCL+30%HA (Fig. 4.1D). For 1 days however, the number of cells in the wells in the presence of SPCL, SPCL+10%HA and SPCL+30%HA was found to be statistically lower comparatively to SCA+20%HA. After 7 days this difference was again noted between SPCL and SCA+30%HA. Contrarily, at the same time of culture in the wells in contact with SPCL+20%HA and SPCL+30%HA the number of cells was statistically higher than in contact with SCA+10%HA.

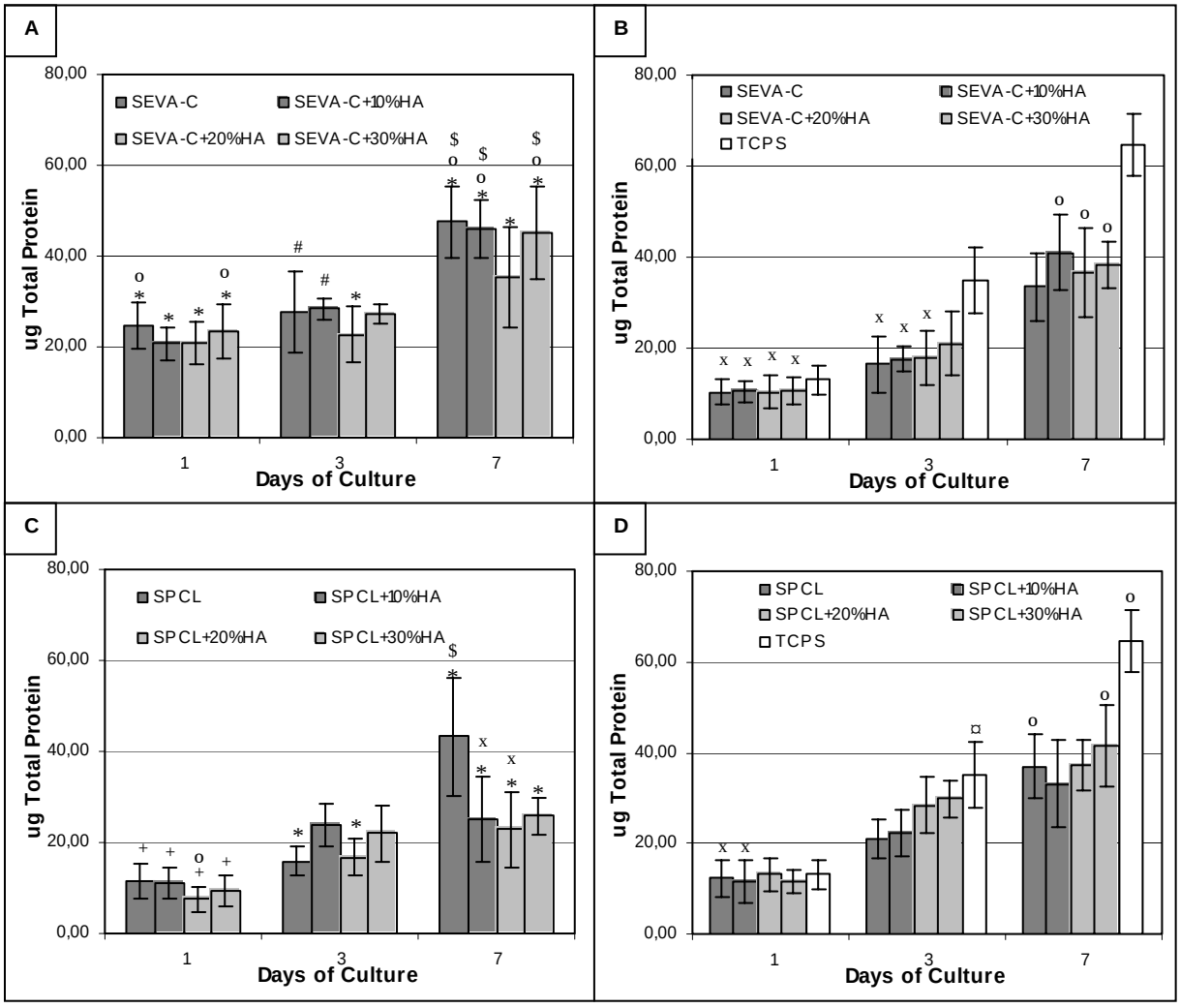
Apparently the effect of the degradation products of the starch-ethylene vinyl alcohol and starch-polycaprolactone blends was not significant and no correlation could be made. Nonetheless, and contrarily to what was found for SEVA-C and respective composites, the amount of total protein in the wells was higher than on the surface of SPCL and respective composites for all the times of culture. Thus, the surface of these starch-based biomaterials does not seem to be preferred over the TCPS wells. Taking into account that after 7 days of culture SPCL composites did not support a higher number of cells than after 3 days, it would be expected that the number of cells on the wells, where those materials were present, would be higher. However the obtained values were comparable to those measured in the presence of the unreinforced polymer (SPCL) which demonstrates that the properties of the surface of the SPCL composites are in fact ruling and delaying osteoblast-like proliferation on its surfaces.

4.3.1.3. SCA and Composites

The number of cells quantified on the surface of SCA and respective composites, as observed for the blend of starch-polycaprolactone, was statistically lower comparatively with SEVA-C and its composites at day 1. At this time of culture no difference was observed comparing to the control TCPS although after 3 days the amount of protein in the starch-cellulose acetate materials, except SCA+10%HA, was already significantly lower. At the end of the assay, SCA and its composites presented a significantly lower adhesion/proliferation on their surfaces comparatively to TCPS. In the third day of culture, no significant differences were observed between the amount of cells quantified on the surface of SCA and its composites and on the surface of the other materials. Differences occurred at day 7 with differences between SEVA-C and respective composites and SCA+30%HA and between SPCL+10% and SPCL+20%HA and SCA+20%HA, which were found to be the SCA composites respectively with lower and higher number of cells at this time point.

As for the starch-ethylene vinyl alcohol blend, the reinforcement of SCA with HA did not have a significant effect on osteoblast-like cells adhesion although SCA+30%HA presented the lowest amount of total protein.

Contrarily to the other two starch-based blends the degradation products of SCA composites had an effect on cell adhesion and proliferation on the wells of the materials. After 7 days of culture for increasing percentages the number of total protein decreased and for the unreinforced material the number of cells was higher in the wells and not on the surface of the materials. Thus, in the case of SCA and composites the reinforcement of the polymer could be favourable for cell adhesion if the effect of the degradation rate and consequently of the degradation products did not mask the effect of the surface properties. Comparatively to the polymer without HA, we would say that the surface properties of the composites are more favourable for osteoblast-like cells adhesion and proliferation since the amount on their surfaces is comparable even in the presence of proliferation delaying molecules.



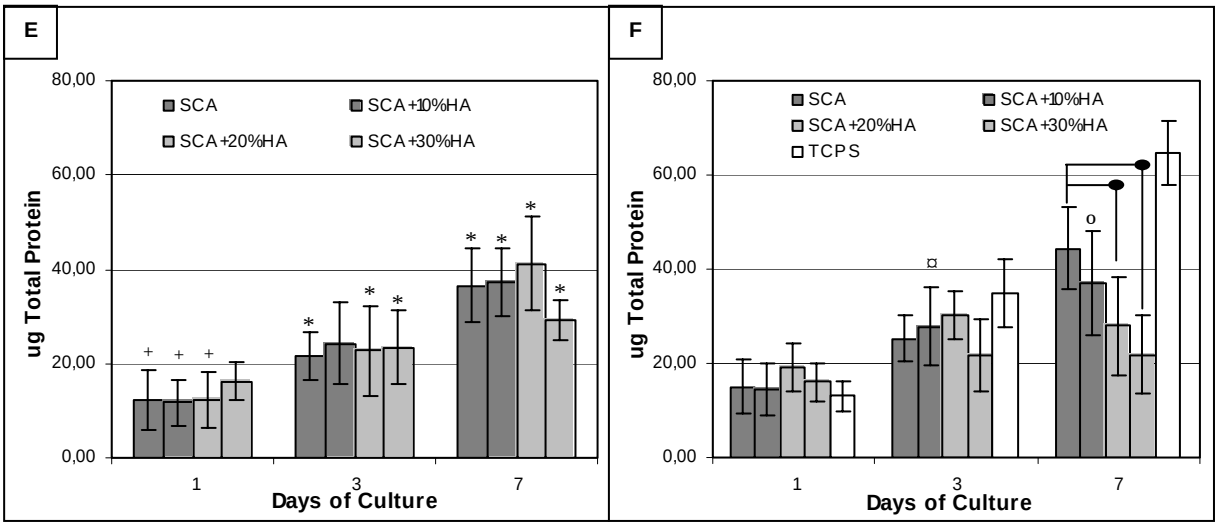


Figure 4.1. Amount of total protein quantified in osteoblast-like cells (SaOs-2) cultured in direct contact with SEVA-C and its composites (A, B), SPCL and composites (C, D) and SCA and its composites (E, F) for 1, 3 and 7 days. Total protein was quantified on the materials (A, C, E) and wells (B, D, F). Data represents mean ± standard deviation, n≥3. *Indicates significant difference when comparing with TCPS (Control). $^{+}$Indicates significant difference when comparing with SEVA-C and SEVA-C composites. $^{¤}$Indicates significant difference when comparing with SEVA-C. $^{\#}$Indicates significant difference when comparing with SPCL. $^{\$}$Indicates significant difference when comparing with SPCL composites. ^{x}Indicates ●— significant difference when comparing with SCA+20%HA. ^{o}Indicates significant difference when comparing with SCA+30%HA. Indicates significant difference between the connected bars.

4.3.2 Surface topography

The morphology of the materials analysed by SEM, showed that SEVA-C possesses a quite irregular surface with some areas of intense irregularities (Fig. 4.2A). At higher magnification it was possible to note that SEVA-C surface is highly asymmetrical with some areas rougher than others although this blend has been reported[33] to be an inter-penetrating network (IPN). After the incorporation of 10% of HA, the surface of the material has become uniform although the presence of HA particles seemed to introduce a rough character to this composite (Fig. 4.2B). The SEVA-C composite with 20% of HA showed again a rather inhomogeneous surface (Fig. 4.2C) that even seemed to have, in comparison with the composite with 10%HA, some smoother areas. The increasing in the percentage of HA incorporated from 20% to 30% did not show significant changes in surface topography (Fig. 4.2D). In fact HA particles are dispersed all over the surface and we might speculate that the difference between those two composites would be the amount of HA granules on the surface. Thus the topography/morphology of the surfaces of the polymers and composites of starch with ethylene vinyl alcohol has changed with the incorporation of HA.

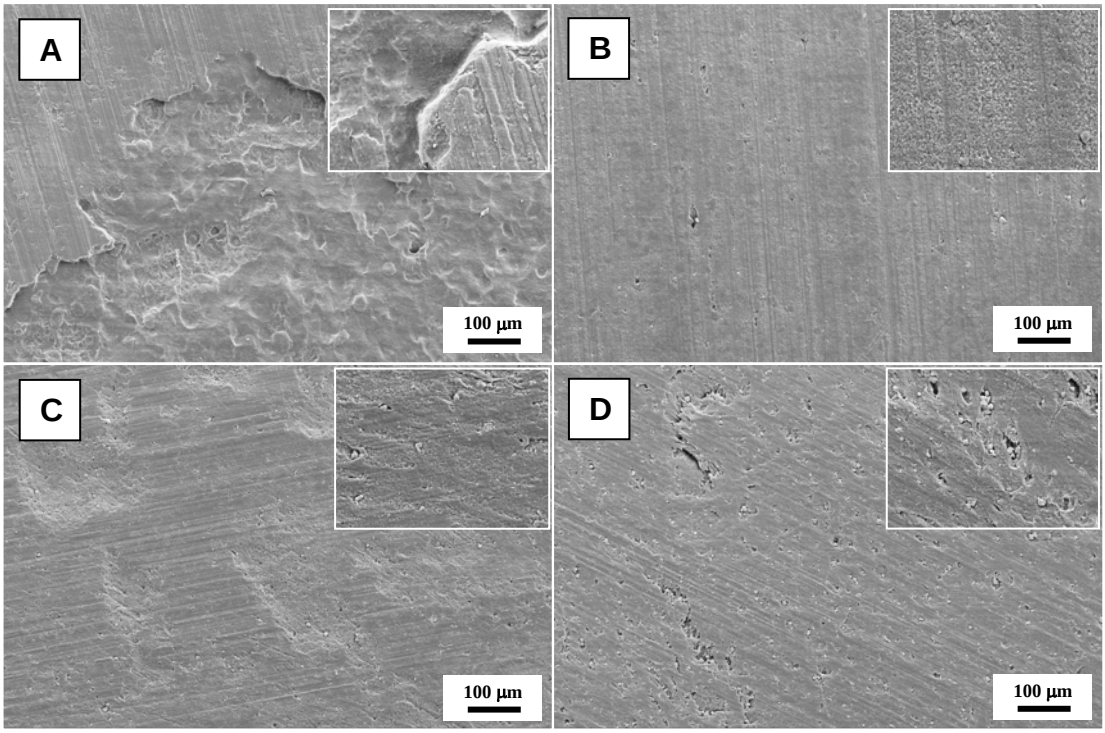


Figure 4.2. Scanning electron micrograph showing the surface topography of (A) SEVA-C, (B) SEVA-C+10%HA, (C) SEVA-C+20%HA, (D) SEVA-C+30%HA; Original magnification x 350. Small squares on the upper corner represent an area of the micrograph at higher magnification (x 1000). Bar = 100 μm.

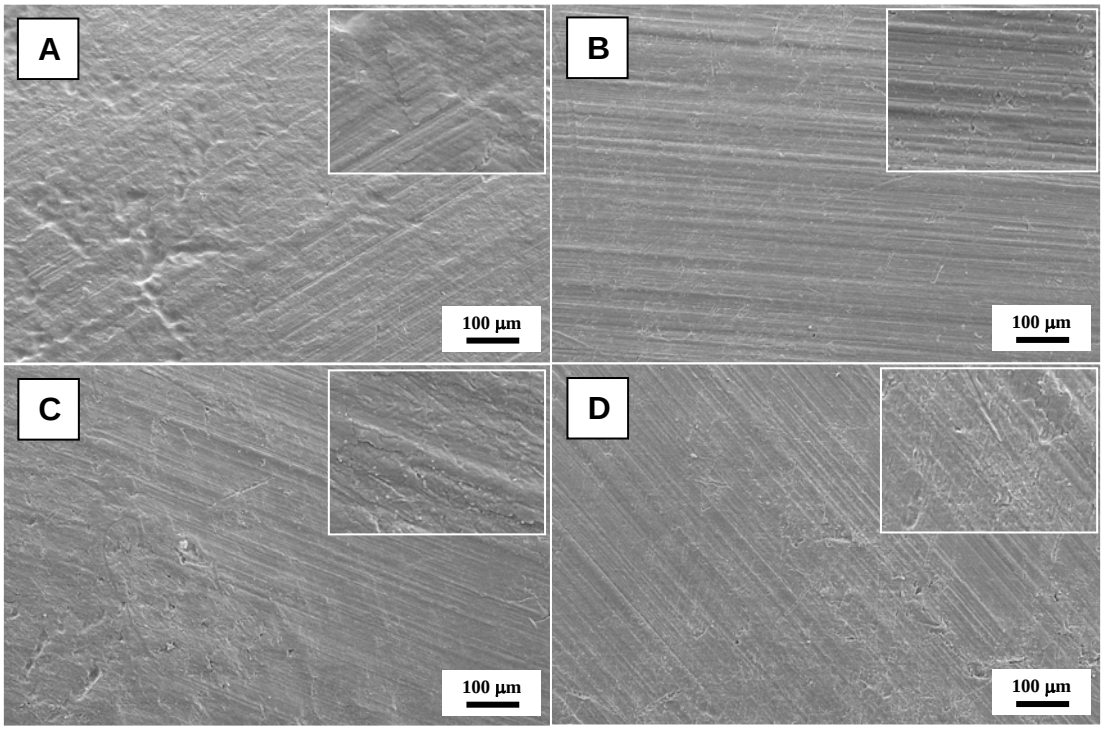


Figure 4.3. Scanning electron micrograph showing the surface topography of (A) SPCL, (B) SPCL+10%HA, (C) SPCL+20%HA, (D) SPCL+30%HA. Original magnification x 350. Small squares on the upper corner represent an area of the micrograph at higher magnification (x 1000). Bar = 100 μm.

Contrarily to the surfaces of SEVA-C and composites, SPCL and SPCL reinforced with HA presented smoother surfaces (Fig. 4.3). In addition, the reinforcement of the SPCL polymer with HA may have introduced some roughness the surfaces of the composites, in particular to SPCL+30%HA. These differences are not notorious and HA particles were not clearly observed on the surface of SPCL composites.

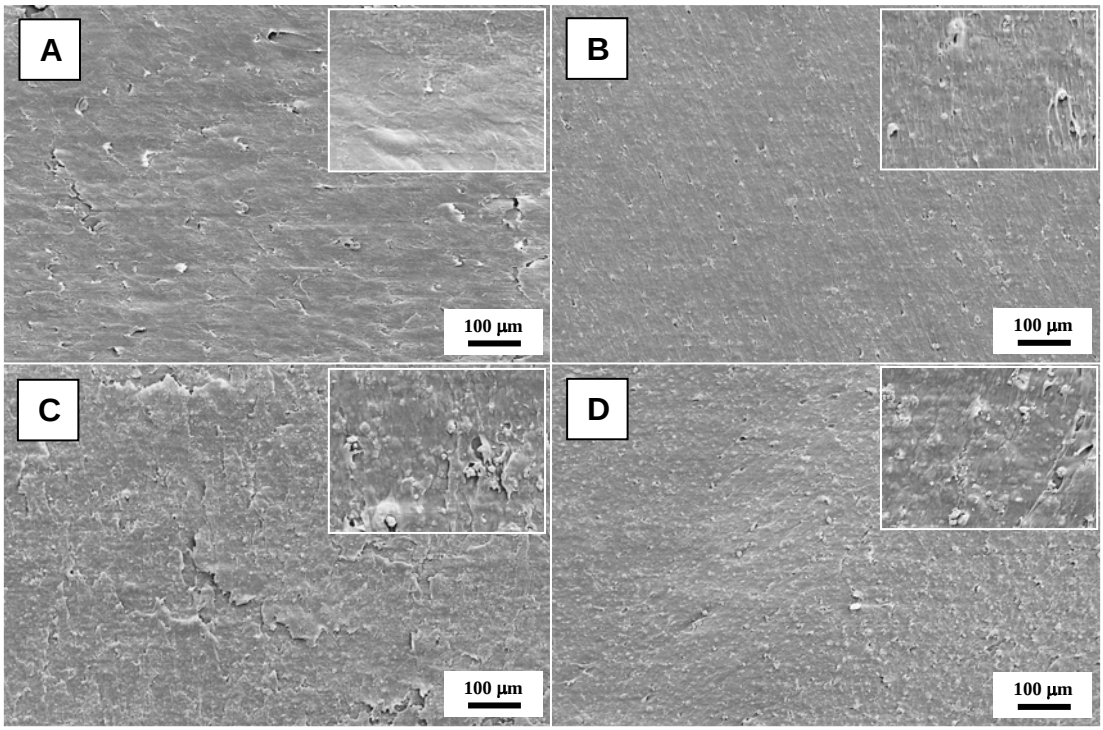


Figure 4.4. Scanning electron micrograph showing the surface topography of (A) SCA, (B) SCA+10%HA, (C) SCA+20%HA, (D) SCA+30%HA. Original magnification x 350. Small squares on the upper corner represent an area of the micrograph at higher magnification (x 1000). Bar = 100 µm.

The morphology of the surface of SCA was not comparable to any of the other starch-based biomaterials (Fig. 4.4A). This material was quite rough and the reinforcement of SCA with HA has resulted, in this blend in a notorious way, in rougher surfaces for increasing percentages of ceramic. Since SCA is the more immiscible blend, the HA particles were visibly present in the surface and in great amounts for SCA+30%HA (Fig. 4.4D).

4.3.3 Cell adhesion/morphology

The adherence of osteoblast-like cells on the surface of starch-based materials was assessed after methylene blue staining. Cells were adhered all over the surface of SEVA-C and composites after 1 and 3 days of culture (Fig. 4.5 A, C, E, G) presenting the typical polygonal shape of osteoblastic cells, therefore showing the suitability of the substrates for adherence.

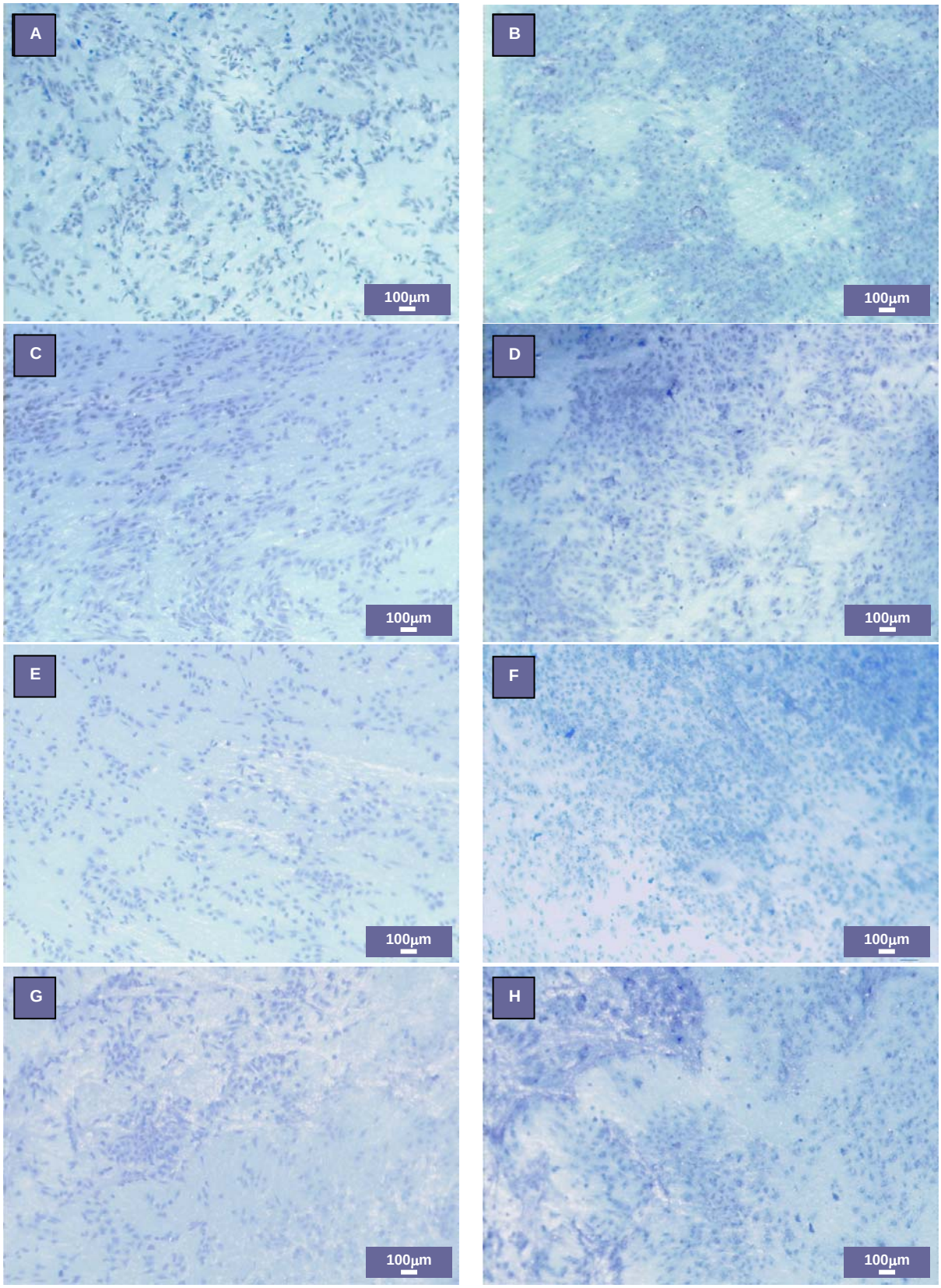


Figure 4.5. Optical micrograph of osteoblast-like cells (SaOs-2) stained with methylene blue cultured on SEVA-C and its composites for 3 and 7 days. Bar = 100µm

The morphology of the cells was analysed in detail by SEM which allowed to see that cells have different morphologies when adhered to SEVA-C comparatively to its composites (Fig. 4.6). After 3 days of culture there were some completely spread cells with extended lamelipodia to the material but also some cells still starting to flatten (Fig. 4.6A). These cells presented filopodia towards the material with some of them already showing lamelipodia. In the case of SEVA-C composites cells were much more spread, highly connected with the surface (Fig. 4.6B). Only few cells were showing filopodia and in the direction of HA particles. However, no significant differences were observed for different percentages of reinforcement. After 7 days of culture, as it was demonstrated by total protein quantification, cells proliferated well on the surface of those materials. In fact some areas of the samples were covered with a monolayer of cells (Fig. 4.5 B, D, F, H), again indicating that SEVA-C and respective composites possess appropriated properties for osteoblast-like cells adhesion. The SEM observation of these surfaces after 7 days of culture proved that cells were completely spread on the surface forming a monolayer. On SEVA-C it was however still possible to distinguish the cell contours while in the case of composites cells were interconnected being impossible to delineate each one of them.

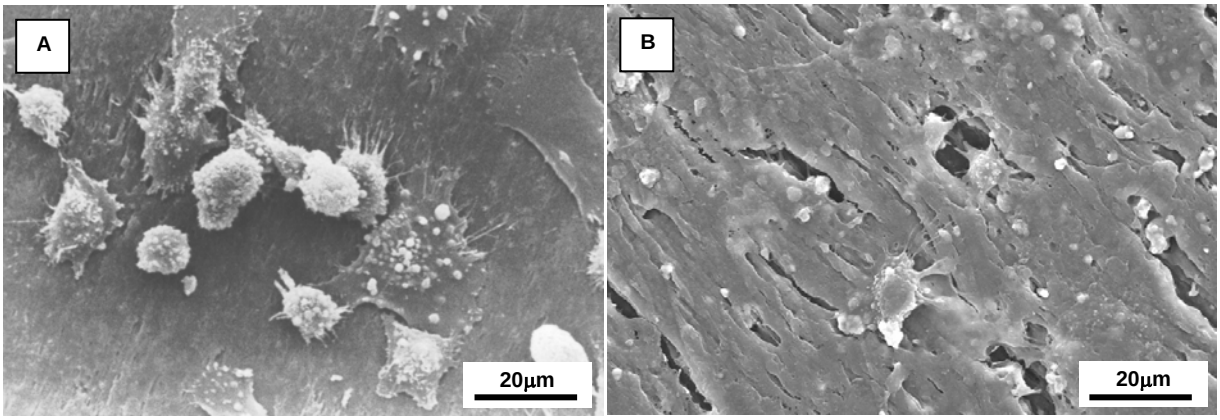


Figure 4.6. Scanning electron micrograph of osteoblast-like cells (SaOs-2) on (A) SEVA-C and (B) SEVA-C+20%HA after 3 days of culture. Bar = 20μm.

The adhesion of osteoblast-like cells on SPCL and respective composites was, at early times of culture and in terms of cell distribution and morphology, similar to what was observed for SEVA-C and SEVA-C composites. Cells seemed to show the typical osteoblastic morphology although this was more obvious for SPCL composites with 20% and 30% of HA (Fig. 4.7 A, C, E, G). SEM evaluation of cell morphology showed that cells on the surface of SPCL and SPCL+10%HA were spread on the surface and merging other cells in the periphery (Fig. 4.8 A, B). These observations were not exactly the same for cells adhered to the surface of SPCL+20%HA and SPCL+30%HA. Cells were also spread, in a higher extent on SPCL+20%HA, but it was possible to distinguish independent cells. Thus increasing

percentages of HA did not seem to favour cell spreading and proliferation on the surface of SPCL materials.

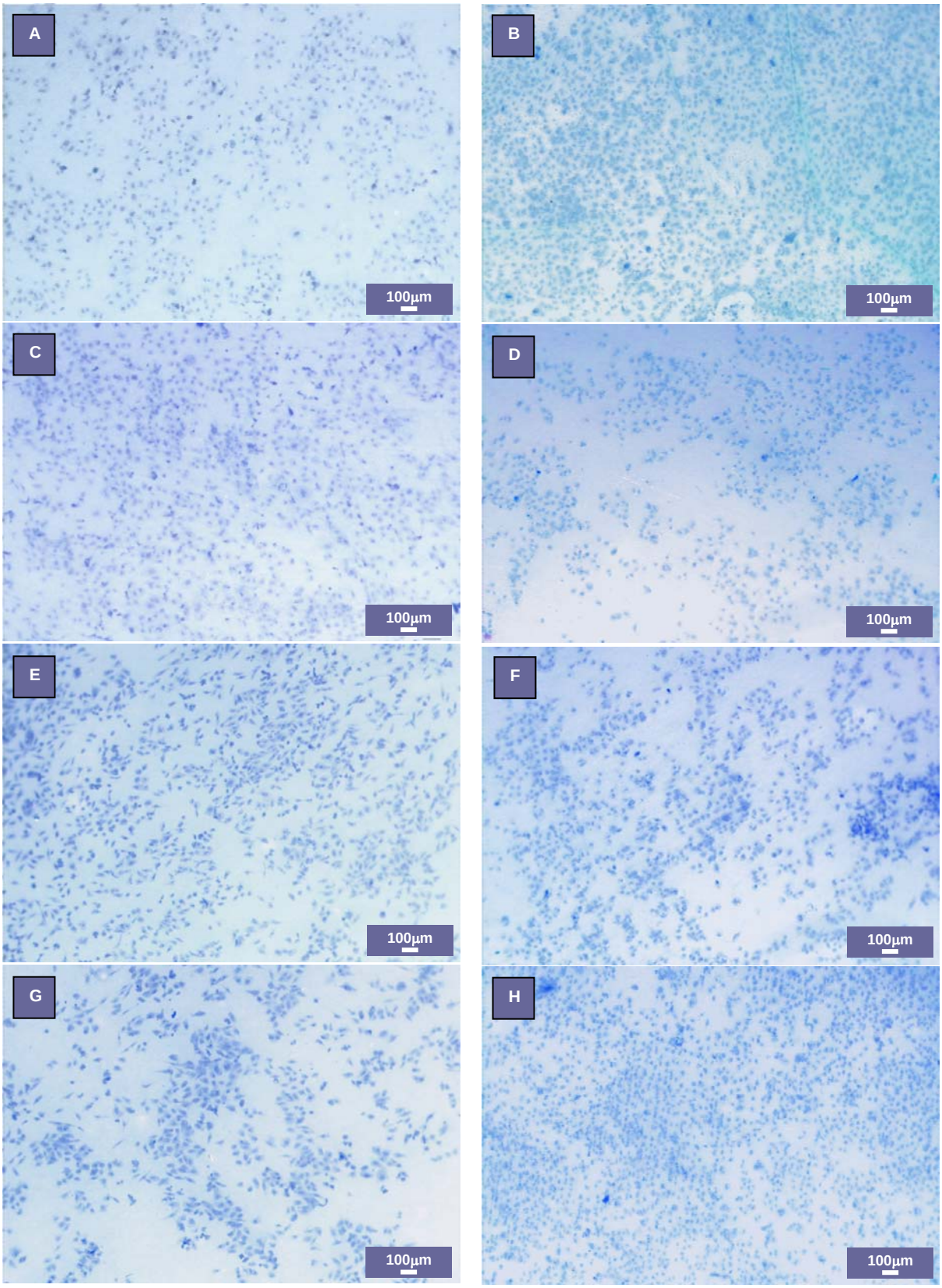


Figure 4.7. Optical micrograph of osteoblast-like cells (SaOs-2) stained with methylene blue cultured on SPCL and its composites for 3 and 7 days. Bar = 100µm

After 7 days of culture, the results obtained for total protein quantification were proved once again. SPCL composites were found not to be the ideal substrate for cell proliferation (Fig. 4.7 D, F, H). Cells were starting to form agglomerates in certain areas of the surface of the composites contrarily to what was observed on the surface of the unreinforced SPCL, which showed cells all over the surface (Fig. 4.7 B). In fact the SEM observation showed that the cells on the surface of SPCL materials were starting to retract after 7 days of culture. The majority of the cells was not spread starting to extend filopodia, which seems to be an attempt to remain attached to the surfaces. Thus, although the surface properties of SPCL and its composites were suitable for initial cell attachment and adhesion, it was found that for increasing times of culture and consequent changes on the surface characteristics as time goes by may render those surfaces improper (less adequate) for osteoblast-like proliferation.

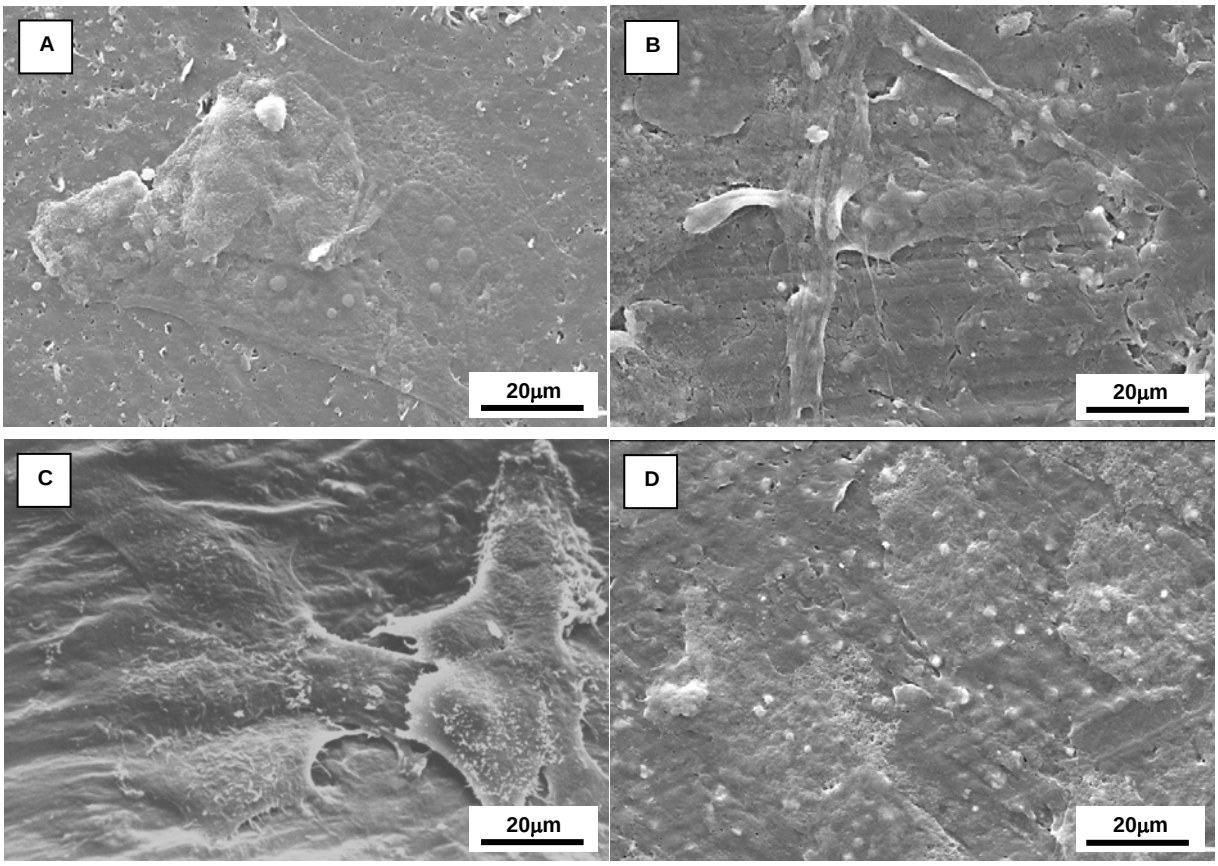


Figure 4.8. Scanning electron micrograph of osteoblast-like cells (SaOs-2) on (A) SPCL, (B) SPCL+10%HA, (C) SPCL+20%HA and (D) SPCL+30%HA after 3 days of culture. Bar = 20µm.

Comparatively to the other blends, the starch-cellulose acetate did not support so well osteoblast-like cell attachment and adhesion. For all the times of culture cells did not present the characteristic morphology of osteoblasts and were preferential adhered to some areas of the surface instead of being all over it (Fig. 4.9). In addition, the differences between SCA and its composites did not seem to be significant.

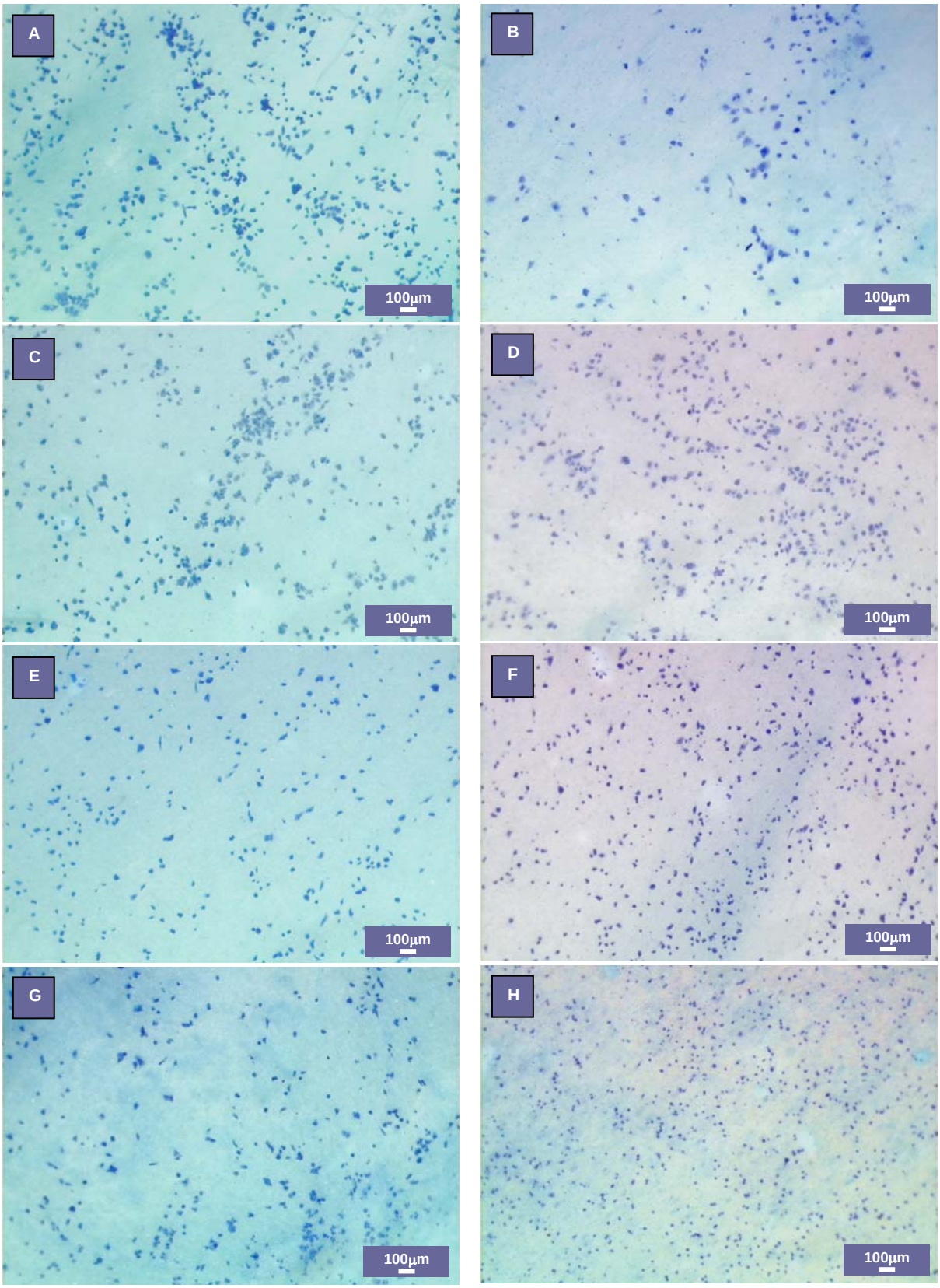


Figure 4.9. Optical micrograph of osteoblast-like cells (SaOs-2) stained with methylene blue cultured on SCA and its composites for 3 and 7 days. Bar = 100µm

The detailed observation of cell morphology confirmed that although adhered to the SCA and respective composites using cytoplasm extensions, the majority of the cells were not spread and its nucleus was prominent and easily identifiable (Fig. 4.10). The amount of spread osteoblasts, comparatively to round cells, on the surface of SCA was still considerable after 7 days (Fig. 4.10A) which may constitute a good sign in terms of suitability of this material for cell adhesion and proliferation. However, for higher percentages of HA, the amount of spread cells decreases (Fig. 4.10 B, C, D). Thus contrarily to what should be expected, in the presence of HA, the morphology of osteoblasts on the surface of SCA composites did not seem to be ideal for cell proliferation.

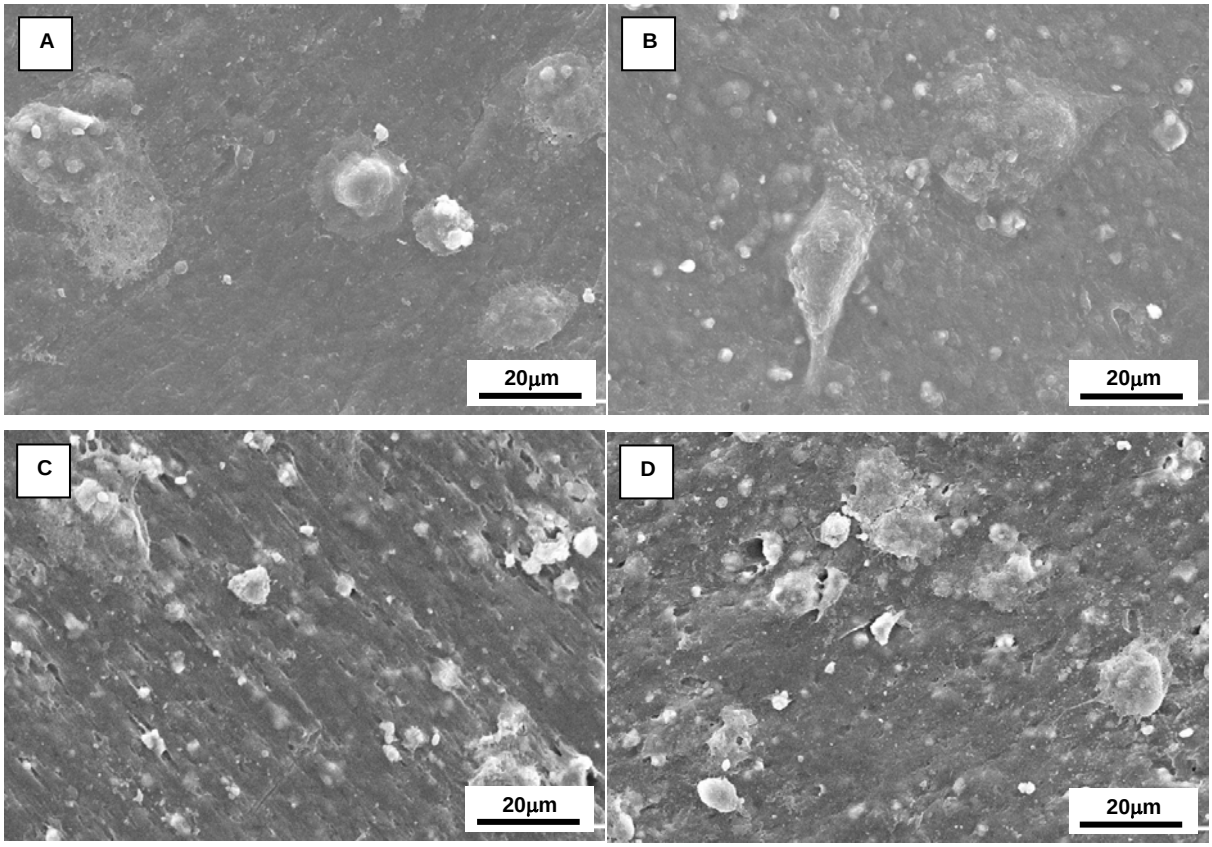


Figure 4.10. Scanning electron micrograph of osteoblast-like cells (SaOs-2) on (A) SCA, (B) SCA+10%HA, (C) SCA+20%HA and (D) SCA+30%HA after 7 days of culture. Bar = 20μm

4.4. DISCUSSION

Surface characteristics of the materials, whether their topography[23,30], chemistry[21,30,34,35] or surface energy[29,36], play an essential part in osteoblasts adhesion to biomaterials. Attachment, adhesion and spreading belong to the first phase of cell/material interaction and the quality of this stage influence the capacity of cells to proliferate and differentiate itself on contact with the implant.[12]

Cell attachment represents the translation of certain physico-chemical events involving the chemical interaction between cells and materials.[12] This is followed by cell adhesion which is

the result of biological processes such as production of extracellular matrix proteins, cytoskeleton proteins reorganisation among others.[12] Cell adhesion and spreading were shown to be clearly distinguishable biological phenomena because substrates that allow cell adhesion do not necessary promote cell spreading[37]. Furthermore, it was previously suggested[38] that surfaces that show good cell attachment at early time points do not necessarily promote cell proliferation or differentiation.

This study was performed in order to determine which of the starch-based biomaterials would be more suitable for the development of biomedical device for orthopaedic applications and bone tissue engineering scaffolding. The effect of the incorporation and of increasing percentages of a ceramic in osteoblast adhesion/proliferation was also assessed for the three starch-based materials. The experiment was set in order to distinguish the effect of the surface properties on the adhesion/proliferation rate of osteoblast-like cells from possible degradation products released from the materials to the culture medium. Furthermore, cell morphology was also analysed and correlated with the topography of the surface of the materials.

Cell growth capacity has been shown be influenced by different chemistries of the materials.[21,30,34,35] For example, hydroxyl groups are known to enhance cell adhesion and growth[39,40]. These groups are responsible for higher surface polarity and hydrophilicity of the surface[13]. However, a correlation between these two parameters has not been a consensus. Some authors[15,41,42] defend that cell adhesion is generally better on hydrophilic surface. However, other studies[11,13,28] showed that osteoblast-like cells do not display a consistent trend of behaviour in relation to surface wettability but rather varied as a function of particular functional groups. Studies[29,36] with osteoblasts suggested that cell adhesion was greatly influenced by the polar interaction energy, which emphasises the role of surface energy in this biological process.

Due to their starch component, the materials in study have high number of hydroxyl groups on their surfaces. In addition SCA is the more hydrophilic material and possesses higher content of oxygen[43]. Thus it would be expected, based on this properties, that the blend of starch with cellulose acetate would have higher number of cells adhered to its surface. However, SEVA-C with the lowest oxygen content and a less hydrophilic[43] surface than SCA presented higher cell adhesion and a regular proliferation rate. SCA is a non miscible blend and due to its higher water uptake capability and degradation rate experiences more and more rapidly changes on its surface which definitely determine and influence cell behaviour.

In addition to chemistry, osteoblasts react differently according to surface topography[23,30] and roughness[4,22,40,44]. Rougher surfaces were shown to reduce proliferation of osteoblast-like[4,44] and human bone derived cells[22,45]. In addition, the initial adhesion of osteoblast-like cells was shown to be greater on polished (smoother) surfaces.[4]

A direct relationship exists between roughness and surface energy of the materials and it was demonstrated that the apolar component of surface energy increased significantly with roughness.[46] Furthermore, it was reported[47] that for relatively low surface roughness values, cell responses to the surface chemistry are more important than the physical surface.

In terms of topography, starch-based biomaterials presented different surfaces apparently showing irregularities that might influence cells adhesion. SPCL polymer seems to have the smoother surface[48]. Therefore, the wettability and roughness of SPCL would indicate that this material did not present the best properties for cell adhesion. However, cells adhered to its surface similarly to SEVA-C which might suggest that roughness plays a more important role than wettability in cell adhesion to SPCL. In addition, the oxygen content of SPCL is similar to SCA[43] and we could suggest that it also has a role in osteoblast-like cells adhesion to SPCL.

After cells contact surfaces they will alter their cell membrane and its morphology to stabilise the cell-material interface[49]. When cell adhesion was followed by progressive flattening of the cells, proliferation occurred[50].

Some studies[11,13,22,51] demonstrated ultrastructural differences in cell spreading and filopodia forming in dependence on a surface even if no differences in the percentage of adherent cells were observed[51]. Filopodia, finger-like protrusions of plasma membrane formed as a consequence of actin assembling in long bundles or lamellipodia if assembled in the form of mesh supporting sheet-like protrusions are morphological details, characteristic of cell adhesion.[12]

Morphological aspects, like cell adhesion and proliferation, have also been shown to be influenced by different chemistries of the materials.[11,13,15,30] A critical value for the surface energy of the substratum above which cell spreading occurs, was previously established.[20] Likewise, cytoskeleton organisation and cell morphology are regulated by surface wettability[11,36]. Cell attachment and spreading are generally greater on certain moderately hydrophilic surfaces relative to hydrophobic ones[11,36].

Surface wettability of starch-based materials definitely influences cell morphology. SEVA-C materials with intermediate hydrophilicity has shown highly spread osteoblast-like cells on its surface while cells on the surface of SCA, the most hydrophilic material, were adhered but not flat or spread. Interestingly enough, the hydrophobic surface of SPCL material supported cell adhesion and spreading for early but not for longer culture times. Osteoblasts are also shown to recognise substrate morphology and to respond by altering their spreading degree[52,53]. Several studies[4,22] have demonstrated that cell spreading and continuous cell layer formation was better on smooth surfaces compared to rough ones. However, Bigerelle et al[54] suggested that topography below the cell scale favours polygonal morphology of osteoblasts although when the topography was considered above the cell scale they also

appreciate the roughness which may explain cells being spread and flattened on surfaces considered rough[52]. Morphologically, cell layer organisation was also modified by the roughness of the underlying substrates[22]. Our results are, in some extent, in accordance with these observations; the rougher material, SCA, showed the lower osteoblast flattening degree. However, on the smoother surface (SPCL) cells were very spread at short culture times but the surface was not able to support a cell layer.

As the surface characteristics determine how proteins adsorb to the surface[55,56] and more particularly determine the orientation of those adsorbed molecules[17,57] proteins constitute another variable in the cell attachment/adhesion process. Fibronectin (Fn) and vitronectin (Vn) have been shown to be involved in osteoblast adhesion *in vitro*[22,58-60] although they preferentially adhere to Fn[59-61]. However, the surface charge or the material might counteract this response[60]. Fn undergoes greater conformational change when adsorbed onto hydrophobic surfaces than on hydrophilic ones while the conformational changes on adsorption of Vn is substrate independent[56-58]. In addition, *in vitro* cell attachment was shown to be primarily mediated by Vn due to its ability to adsorb to the substrate in competition to other serum proteins.[19,58,62] Attachment to TCPS *in vitro* also depends on Vn adsorption.[62]

A previous study[33] with starch-based polymers and protein adsorption from serum showed that vitronectin is the protein that adsorbs in higher amount to those materials. Furthermore, SPCL was the material with higher amount of adsorbed Vn in comparison with SEVA-C and SCA[33]. Thus, it is likely that this protein plays a major role in the initial cell attachment to SPCL. The surface properties determine that initially Vn, when adsorbed onto this polymer will adopt a conformation that is ideal for cell attachment which becomes less favourable or desorbs from the surface with increasing periods of culture.

Bone has been shown to mechanically react to an HA surface *in vivo*[14]. However, the *in vitro* attachment and growth of osteoblast cells on HA ceramics or other biodegradable polymer reinforced with it has been reported[3,9] to be significantly low compared to a range of orthopaedic biomaterials.

When osteoblasts were cultured with HA particles, the cell population was significantly decreased[63]. Fine particles of HA, normally a non-toxic material were shown to cause cell damage *in vitro*[64], which depend on the direct contact between cells and particles resulting in cell membrane damage. On the other hand, the test material may have a low level of toxicity which, although not sufficient to kill cells, may inhibit normal cell function. The intracellular dissolution of calcium-containing crystals was also proved to greatly influence cell behaviour[65,66] Osteoblasts have been implicated in calcium-phosphate degradation[2,67,68] which lead to a significantly inhibition growth. The explanation suggests that the presence of HA particles and its intracellular solubilisation could adversely affect homeostatic

mechanisms and mechanical regulators of DNA synthesis can be modified without any expression of cytotoxic effect[2].

In another study[69], the degradation of hydroxyapatite powders was also associated with poor cellular response but in this case the effect was attributed to an increase in the amount of impurity ions released. High phosphate ions concentration released from ceramics has also been suggested as being inhibitory to cell activity[5].

Previous short and long-term studies[70-72] with SEVA-C-based HA composites and different types of cell lines have shown promising results which were confirmed in this study. The release of HA particles during the experiment may only constitute an explanation for SCA composites since it was only with these materials that the proliferation rate on TCPS was kept or reduced and only for longer times of culture. In fact SCA is the material with higher water uptake capability and a higher access to the inner HA particles within the composite and a higher susceptibility to hydrolysis at the interface polymer-HA comparatively to the bulk of the material. The easier access to those interfaces facilitates the degradation of the material with the release of not only HA particles but also low molecular weight chains responsible for a pH drop. Thus, for the periods of time tested the amount of released HA particles may induce some inhibitory activity on the osteoblasts.

Furthermore, the surface properties of SPCL composites were shown to be inappropriate for cell proliferation. In this particular case, HA particles itself do not seem to be responsible for this behaviour. In turn, the incorporation of the ceramic seemed to have affected the surface properties in such way that between 3 and 7 days osteoblast decreased its proliferation rate.

The composition and topography of HA composites influenced the morphology of cells, showing that cell spreading was more pronounced on exposed HA regions of the composite[73]. In addition, human osteoblasts showed propensity for spreading at early time points on surfaces containing exposed HA particles[73].

A slow rate of osteoblast-like proliferation on HA as well as weak affinity of fibronectin to that ceramic has been previously reported[74] and associated with the physicochemical characteristic of the material. In another study[31], vitronectin and fibronectin were not only found to adsorb to HA but also to participate in the osteoblast spreading on that material.

In this work one could confirm that, in comparison to unreinforced polymer, starch-based composites induced more pronounced cell spreading. The miscibility character of each one of the starch-based blends also determines the exposure of the HA particles within the samples. Thus, SCA as the more immiscible blend and the more hydrophilic material, presented higher amount of HA on its surface and higher access to the HA particles in the bulk of the composite, was expected to show higher spreading of osteoblasts. However, the spreading of osteoblasts on SCA composites was not as notorious as on SEVA-C and SPCL composites.

4.5 CONCLUSIONS

The results reported in this study indicate that the physico-chemical properties of starch-based biomaterials influenced adhesion, proliferation and morphology/spreading of osteoblast-like cells. Depending on the starch blend, thus on its synthetic component and the properties that it confers to the surface, cells proliferate at different rates. Furthermore, the incorporation of hydroxyapatite also had different effects according to the polymer matrix used. In the case of SCA it seemed to change its degradation behaviour and consequently the degradation products released to the culture medium which delayed cell proliferation. In the case of SPCL, the incorporation of HA induced changes in the surface properties that induced cell detaching for longer culture times. Different percentages of HA did not seem to change significantly osteoblast-like cell behaviour.

Overall results indicate that starch-based biomaterials present characteristics of cell adhesion/spreading and proliferation that are not disappointing considering their degradable nature. In fact as shown in other works these polymers and composites may find several applications in orthopaedics and tissue engineering scaffolding.

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SECTION III

IMMUNOCOMPATIBILITY OF STARCH-BASED BIOMATERIALS: POLYMERS AND COMPOSITES

CHAPTER 5

EVALUATION OF THE POTENTIAL OF STARCH-BASED BIODEGRADABLE POLYMERS IN THE ACTIVATION OF HUMAN INFLAMMATORY CELLS

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ABSTRACT

The inflammatory response resulting from the implantation of a medical device may compromise its performance and efficiency leading, in certain cases, to the failure of the implant. Thus, the assessment of the behaviour of inflammatory cells *in vitro*, constitutes a key feature in the evaluation of the adverse potential, or not, of new promising biomaterials. The objectives of this study were to determine whether starch-based polymers and composites activated human neutrophils.

Blends of starch with ethylene-vinyl alcohol (SEVA-C), with cellulose acetate (SCA) and polycaprolactone (SPCL), as well as composites based on all these materials filled with hydroxyapatite (HA) have been studied. A lysozyme assay was adapted to examine enzyme secretion from human neutrophils incubated with different starch-based materials. Changes in the free radical and degranulation activity of the neutrophil were also determined by measuring the luminescent response of Pholasin®, a photoprotein that emits light after excitation by reactive oxygen species. The amount of lysozyme secreted by neutrophils incubated with the polymers did not exhibit significant differences between the tested materials. Results were in all cases similar to those obtained for the control (polystyrene) except for one of the starch blends (corn starch with polycaprolactone reinforced with 30%(w/w) of HA).

The chemiluminescence experiments showed that polymers reduce the signal produced by activated neutrophils. Furthermore, for some polymers it was demonstrated that the phenomenon was due to an effect of the surface of the materials in cell adhesion or a simultaneous competition for the photoprotein in solution, which results in the decrease of the intensity of light emitted and detected.

5.1. INTRODUCTION

Following the implantation of any medical device, the wound healing mechanisms are triggered in response to injury and to the presence of a foreign body. The inflammatory response constitutes one of the stages of that complex process aiming to eliminate the cause of injury and any accompanying micro-organisms and to initiate the repair of the surrounding tissues. Biomaterials are not totally inert to the surrounding tissues and thus, an inflammatory reaction is produced by any biomaterial, the severity and duration of which can vary according to the properties of the material.[1]

Polymorphonuclear leukocytes (PMNs) are the first cells to arrive at the implant site after surgery. They play a very important role in host defence processes being stimulated by a variety of agents. Their activation may result in several processes such as chemotaxis, phagocytosis, degranulation and production of O2- in a metabolic event known as respiratory burst[2,3].

Degranulation of neutrophils causes the release of granule contents into the surrounding tissue, which contain human neutrophils peptides, also known as defensins[4]. Defensins perform intracellularly by permeabilising and killing microorganisms[5] and outside the phagocytic vacuoles by acting as a chemotaxin for monocytes and lymphocytes[6,7]. Lysosomes contain numerous types of enzymes that are secreted into the tissues during degranulation and frustrated phagocytosis causing severe injury.[8] Neutral proteases such as elastase and collagenase, acid hydrolases and lysozyme are some examples.[9-11]

Together with degranulation the microbicidal activity of neutrophils can result from mechanisms dependent on oxygen.[12] The oxygen-dependent mechanisms consume oxygen as an electron acceptor in reaction initiated by the activation of a multicomponent electron transfer system, the NADPH-oxidase.[13] In this way toxic unstable superoxide anions are produced which can be dismuted by superoxide dismutase to antimicrobial hydrogen peroxide.[14]

A massive and generalised activation of leukocytes may however, impair the host by the excessive release of oxygen radicals and enzymes. This response by leukocytes in the presence of biomaterials can be considered an important measure for biocompatibility for this reason alone. The factors that minimize inflammation will maximize biocompatibility.[15] The multiple responses possible during leukocyte activation and an incomplete understanding of their interactions, lead to the need to measure more than one response to characterise the extent of activation.

Starch-based biodegradable polymers and composites have been proposed for several biomedical applications[16-19]. Biocompatibility studies were already made[20-22] and the aim of

this work was to evaluate the potential of these materials in the activation of human inflammatory cells using two complementary techniques.

Neutrophils were isolated from peripheral human blood and challenged, *in vitro*, with different starch-based polymers and composites. The amount of lysozyme released from neutrophils after incubation with the materials was quantified by means of an assay previously adapted for this type of evaluation.[23] The oxidative burst of neutrophils in the presence of the materials was measured by chemiluminescence. Two cell stimulants, formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-myristate-acetate (PMA) were used as positive controls for both assays.

5.2. MATERIALS AND METHODS

5.2.1 Materials

The materials studied were: i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C, Novamont, Italy), ii) SEVA-C reinforced with 10%, 20% and 30% (wt) of hydroxyapatite (HA, Plasma Biotal, UK), iii) a 50/50 (wt %) blend of corn starch and cellulose acetate (SCA, Novamont, Italy), iv) SCA reinforced with 10%, 20% and 30% (wt) of hydroxyapatite, v) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL, Novamont, Italy) and vi) SPCL reinforced with 10%, 20% and 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

Poly-L-Lactide (PLLA, Purac biochem bv, The Netherlands), being the gold standard for biodegradables in biomedical applications, was used as a biodegradable control material and borosilicate glass coverslips (BDH, England) for chemiluminescence tests.

All the materials, both the polymers and the composites were processed into circular samples (∅ 1cm) by injection moulding.

5.2.2 Neutrophil Isolation

Neutrophils were isolated from fresh heparinised peripheral human blood collected from healthy volunteers. Blood was mixed with a 6% dextran solution, settled and the supernatant layered onto lymphocyte separation medium and centrifuged at 2400 rpm for 25 minutes at room temperature. The pellet was washed once with phosphate buffered saline (PBS) solution without calcium and magnesium and the remaining red blood cells were removed by water lysis. The cell suspension was washed twice with PBS without calcium and magnesium at 2400rpm for 5 minutes at room temperature. Cells were counted and kept at 4°C until use.

5.2.3 Lysozyme Assay

The isolated cells were resuspended in PBS with calcium and magnesium at a concentration of $1.5x10^6$ cells/ml. Each material was incubated in polystyrene cuvettes with 1ml of cell suspension for 30 minutes at 37°C. A cuvette with cells alone was also used as the negative control. After incubation, the tubes were centrifuged at 2400 rpm for 5 minutes at 4°C and 0.5 ml of the supernatants transferred to new tubes to which was added 0.5ml of Micrococcus Lysodeikticus (1.5mg/ml). These tubes were incubated for 30 minutes at 37°C. The lysozyme released by the neutrophils when in contact with the materials breaks down the cell wall of Micrococcus Lysodeikticus reducing the optical density of the suspension, which can be recorded using a spectrophotometer at a wavelength of 541nm. In order to quantify lysozyme secretion, a standard curve was prepared with dilutions of neutrophil lysate versus absorbance readings.

5.2.4 Chemiluminescence

Chemiluminescence is often used to study the neutrophil respiratory burst resulting from biomaterial interactions.

Changes in the free radical and degranulation activity of the cells were measured by the luminescent response of Pholasin® a photoprotein that emits light after interaction with the reactive oxygen species.

The isolated neutrophils were ressuspended in PBS without calcium and magnesium at a concentration of $1x10^6$ cells/ml. Two luminometer cuvettes were prepared for each material with 390μl of HBSS/Hepes buffer, 100μl of Adjuvant-P™ (Abel® Cell Activation Test, Knight Scientific, UK) and 100μl of cell suspension. Two tubes with 100μl of buffer instead of cells were prepared as negative control and another two without any material to work as control in order to verify the activation potential of the cells. All the cuvettes were loaded into the luminometer (Luminometer 1250, LKB Wallace). The first cuvette was then automatically moved to the measuring chamber and 250μl of Pholasin® (Abel® Antioxidant Test, Knight Scientific, UK) injected, followed by successive cuvettes. The light output from the solution was measured every 250 seconds for 33minutes. At this point, 80μl of formyl-methionyl-leucyl-phenylalanine (fMLP, 12μML-1) were injected into each cuvette in succession and the light emitted measured for 10 minutes after which 80 μl of phorbol-myristate-acetate (PMA, 8μML-1) were injected into each tube. Measurements were then taken measuring the light emitted by each cuvette for a total time of 80 minutes.

Peak luminescent values were determined plotting the amount of light emitted per second (mV) versus time.

5.2.5 Evaluation of polymers potential to quench light

In order to assess the effect of the materials themselves on the reduction of the signal produced by activated neutrophils an antioxidant assay was performed in the absence of cells but in the presence of free radical donor reagents.

Two replicates of each tube were prepared with 1.335ml of HBSS/Hepes buffer and 125μl of Pholasin® and loaded into the luminometer. After 2 minutes, 40μl of 3-morpholino-sydnonimine HCl (SIN-1, 2.5mmolL^{-1}, Abel® Antioxidant Test, Knight Scientific, UK) were injected to each tube. Superoxide and nitric oxide are simultaneously and continually released from the solution of SIN-1. If there are material interactions with the free radicals there will be a delay in the time at which the maximum peak of light is emitted or the magnitude of the peak. Empty cuvettes were the negative control.

5.2.6 Statistical Analysis

All data was averaged and standard deviation is reported as a measure of sample deviation. The effect of the tested materials on the release of lysozyme was compared statistically with Tukey-HDS test.[24] All the materials were compared between themselves and the control. If probability values were less than 0.05 ($p<0.05$), differences observed for the two materials were considered statistically significant.

5.3. RESULTS AND DISCUSSION

5.3.1 Lysozyme

Polymorphonuclear leukocytes comprise one of the immune systems first lines of defence through phagocytosis and destruction of microorganisms. Stimulated phagocytes release lysozomal enzymes and produce a large amount of superoxide anion with the secondary generation of more oxidant species, which result in non-specific damage to surrounding tissues and varying degrees of inflammation.

Several factors influence the phagocytic activity at a biomaterial's interface. It has been shown that the human PMN respiratory burst is influenced by the adhesion to a surface and by the wettability of that surface in the presence or absence of proteins.[25-30]

Lysozyme was released by neutrophils after incubation with the degradable materials in study; this was less than 20% of the potential lysed cell maximum for all of the materials (Fig 5.1). Furthermore, neutrophils incubated with SEVA-C, all SEVA-C composites, SPCL, SCA reinforced with 10%HA and PLLA did not secrete lysozyme above the negative control (polystyrene test tube). SPCL composites stimulated more enzyme secretion and in fact, the results obtained for SPCL reinforced with 30% of HA are statistically different from those

obtained for SEVA-C polymer, all their composites and for PLLA and the control. Also for SPCL with 20% of HA and SEVA-C with 30% of HA, the difference in the amount of lysozyme secreted was significant at the level of 0.05.

The amount of hydroxyapatite in each composite does not correlate with the amount of enzyme secreted in the presence of SEVA-C composites. However, in the case of SPCL, for higher percentages of HA, the quantity of lysozyme released tends to increase. The same type of behaviour was observed for SCA composites, although in this case the polymer without any reinforcement induced an even higher percentage of enzyme secretion than the composite with 30% of HA. This seems to indicate that the observed behaviour depends on the SCA matrix and when its amount is reduced becomes less intense.

Higher responses have been detected after PMN interaction with hydrophilic surfaces *in vitro* and *in vivo*[26,31] but in the presence of human serum, neutrophil adhesion and activation is triggered on hydrophobic surfaces *in vitro*[27].

SPCL, SCA and their composites seem to give rise to higher secretion of lysozyme than SEVA-C and its composites. As SPCL and SCA have extreme wettability properties, they are respectively the most hydrophobic and the most hydrophilic of the starch based blends (with a water contact of 70° and 55°, respectively). In fact, the incubation with SCA resulted in higher enzyme secretion, which can be a consequence of a higher cell interaction and activation previously reported for hydrophilic surfaces[26,31].

One might speculate that SCA composites would induce the highest neutrophil response. The capacity of those materials to uptake water is higher than the polymer without reinforcement due to the interfaces between polymer and HA particles. In fact it has been shown that there is usually a preferential absorption of water and consequent degradation at starch/HA interfaces when its processing is not fully optimised.[32] Thus these particles due to their size, comparing with the matrix, would constitute a preferential site for adhesion, phagocytosis with neutrophils experiencing high levels of degranulation.

PLLA was used as a comparison biodegradable material due to it extensive applications in the biomedical field. It was then possible to observe that the results obtained for starch-based materials were not different from those obtained for the PLLA except in the case of SPCL with 30% of HA. These results indicate that almost all of the starch-based biomaterials (polymers and composites) disclose a behaviour at least as good as that of the actual gold standard in the field.

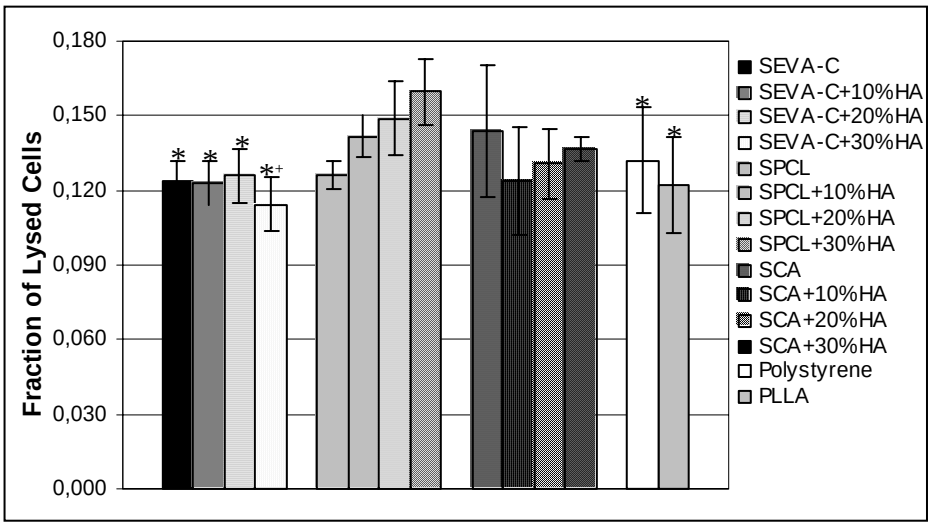

Figure. 5.1 Fraction of Micrococcus Lysodeikticus lysed by the lysozyme released by neutrophils incubated with starch-based materials and composites.
* Statistically different from SPCL+30%HA ($p < 0.05$), Tukey-HDS test.
+ Statistically different from SPCL+20%HA ($p < 0.05$), Tukey-HDS test.

5.3.2 Chemiluminescence

Neutrophil activation may be either due to a direct effect of the material on the cell membrane or mediated by the adsorption of plasma and matrix proteins.[33,34]

The chemiluminescence assay was used to evaluate the potential of neutrophils to become activated after a direct contact with several biodegradable polymers and composites. The light resulting from the interaction of free radicals and other oxidants, produced by stimulated neutrophils, with the photoprotein Pholasin® was detected and plotted against time. After injection of each cell stimulant, fMLP and PMA, a peak for light emission was observed, as expected, due to an increase in the production of oxygen intermediates. Those two chemicals have two distinct mechanisms of action that explains the differences in the intensity of the peaks of light. The receptor stimulant fMLP works via receptors and acts, in most cases, solely on the NADPH oxidase system of the plasma membrane whereas PMA enters the cells acting directly on protein kinase C, which leads to the activation of the NADPH oxidase both on the plasma membrane and on the secondary granules.

From the results shown in Fig 5.1 and Fig 5.2, it is clear that the maximum response in the chemiluminescence tests was significantly reduced when the cells were exposed to polymers (Fig 5.2 and Fig 5.3). At the moment of the injection of Pholasin® into the luminometer cuvettes the slight increase in the light detected expected, was not observed for some materials, which seems to show that the phenomena responsible for the reduction of signal is occurring at an early stage of the assay. Furthermore, the response after the injection of the

cell stimulants was very low for fMLP and reduced for PMA, about 20% when compared with the positive control.

A mechanism for the down regulation of PMN function was demonstrated in a study by Hansch et al[35], which gave evidence that the dialysis-membrane-associated L-fructose residues participate in a complement–independent neutrophil activation during hemodialysis therapy. This monosaccharide was found to be present in cellulose-based polymers in picomolar concentrations.[36]

Moore et al[37] related cellulose acetate degradation with PMN activation *in vitro* and also found that glycerol suppresses reactivity before stimulant addition and after stimulation of neutrophil activation by fMLP or PMA *in vitro* in a dose-dependent manner.

Additionally, it is known that glycerol is used in the manufacturing of starch-based material. This compound may serve to mask a more active inflammatory response to the materials in study since it is the first compound together with low molecular weight chains to leach out from the materials.

It was not possible to observe a tendency in the results either in the case of SEVA-C and composites (Fig 5.2) or in the case of SPCL and composites (Fig 5.3). It is important to point out the result obtained for PLLA, as it was similar to the negative control.

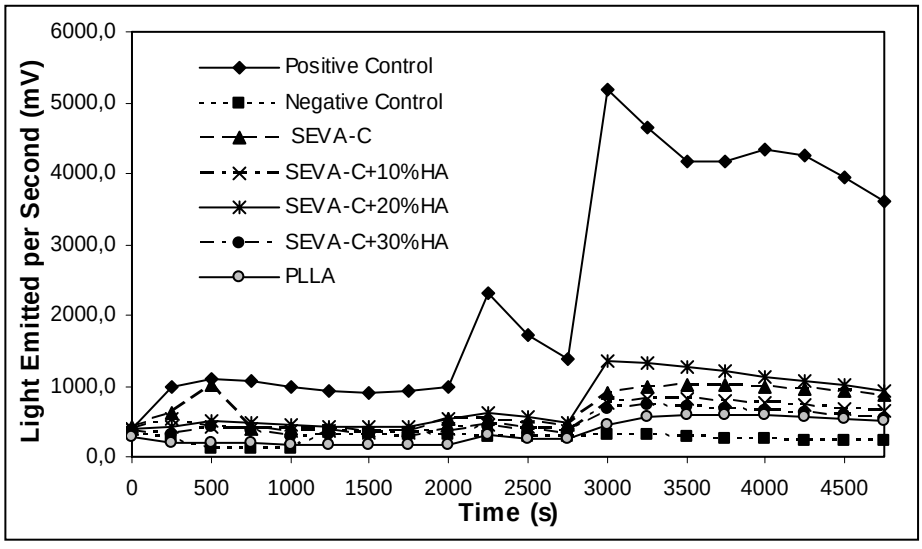

Figure 5.2. Chemiluminescence measurements of respiratory burst of neutrophils exposed to a starch-based polymer (SEVA-C) and composites and to PLLA. Positive control corresponds to the polystyrene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the luminometer cuvette without cells and buffer instead. Two cell stimulants, fMLP and PMA, were injected to all the cuvettes at different times. Graphs represent mean of n=4 separate experiments, with 2 replicates of each sample in each experiment.

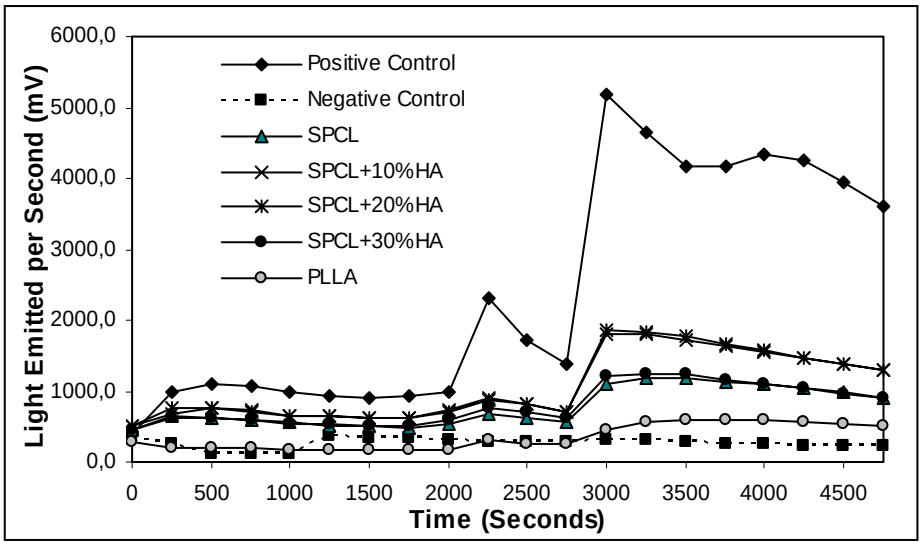

Figure 5.3. Chemiluminescence measurements of respiratory burst of neutrophils exposed to a starch-based polymer (SPCL) and composites and to PLLA. Positive control corresponds to the polystyrene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the polystyrene tube (luminometer cuvette) without cells. Two cell stimulants, fMLP and PMA, were injected to all the cuvettes at different times. Graphs represent mean of n=4 separate experiments, with 2 replicates of each sample in each experiment.

To verify that the time of the assay was not a limiting factor, the assay was prolonged up to 4 hours, but no further changes in the oxidative response of the cells was detected (data not shown).

High levels of Mac-1 expression have been described after contact with surfaces of polymeric materials, which led to increased adhesiveness to the surfaces with a consecutively evoked oxidative burst.[38,39] However, studies ex vivo in a murine model showed that exudate cells respond more to PMA than implant associated cells.[25,40]

To assess whether or not this reduction in the light detected was a consequence of a cell/material interaction or due to possibly free radical interactions with the material, quenching of the light by the material or inhibition of Pholasin®, two types of experiments were carried out.

Firstly, the initial chemiluminescence assay was time-changed. The first light detected in the initial test was almost 4 minutes after the cuvettes being loaded into the luminometer due to the fact that the luminometer carrousel has to move to the measurement position. Considering this, the number of materials to be tested in each experiment and the number of replicates was reduced, which decreased the analysis time. The results are presented in Fig 5.4; it was possible to observe higher differences between the responses obtained with the different materials and a reduction of signal by polymers. Glass was introduced in this test as a new variable trying to see if different results were obtained for non-polymeric materials. In fact, and as expected, the results were closer to the positive control.

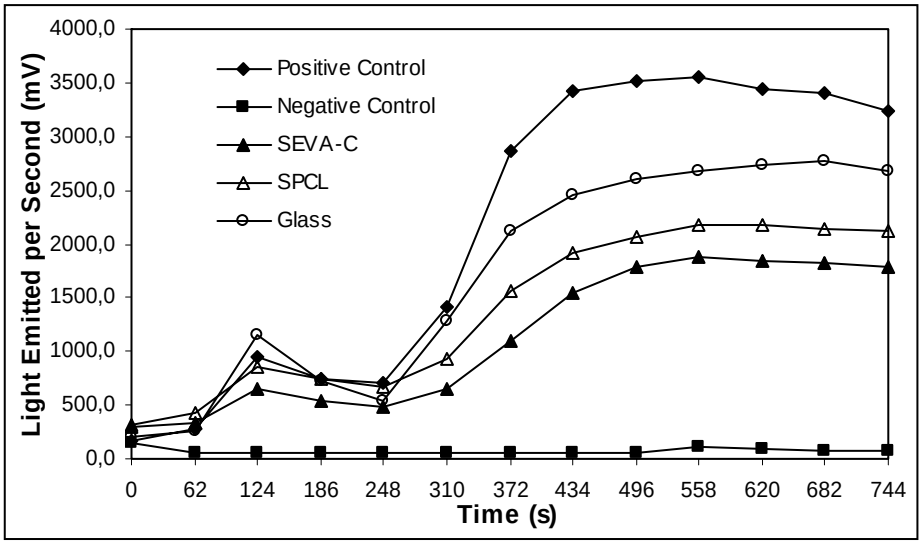

Figure 5.4. Chemiluminescence measurements of respiratory burst of neutrophils exposed to a two starch-based polymer (SEVA-C and SPCL) and to glass. Positive control corresponds to the polystyrene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the luminometer cuvette without cells and buffer instead. Two cell stimulants, fMLP and PMA, were injected to all the cuvettes at different and earlier times. Graphs represent mean of n=3 separate experiments.

The differences are the greatest when cells were stimulated by PMA especially in the case of glass and SPCL. Cells in contact with those two materials presented a similar response in the beginning of the assay becoming different after fMLP stimulation. SEVA-C, on the contrary, was always the material with the lowest intensity of emitted light.

This same experiment was repeated with 2 minutes delay in order to verify if the loading and setting time were crucial for the results obtained in the initial chemiluminescence assay. The results are not shown herein but once again changes were detected. For cuvettes with glass and control the same intensity of light was detected but for the tested polymers (SEVA-C and SPCL) the peak of light after PMA stimulation was lower. The same type of kinetics was observed until this point.

Secondly, nitric oxide and superoxide were released simultaneously and continually from a solution of SIN-1.Those two products reacted forming peroxynitrite that interacts with Pholasin® in the assay. Light of gradually increasing intensity is detected, reaching a peak after a few minutes (Fig. 5.5). The results confirm that the materials or any antioxidants capable of scavenging peroxynitrite are competing for Pholasin®, which results in a peak of lower intensity than the control. Furthermore, when comparing the results for SEVA-C and SPCL, as they are similar, the previous differences obtained (Fig. 5.4) seem to be due to an effect on the adhesion of neutrophils to the surface of those polymers.

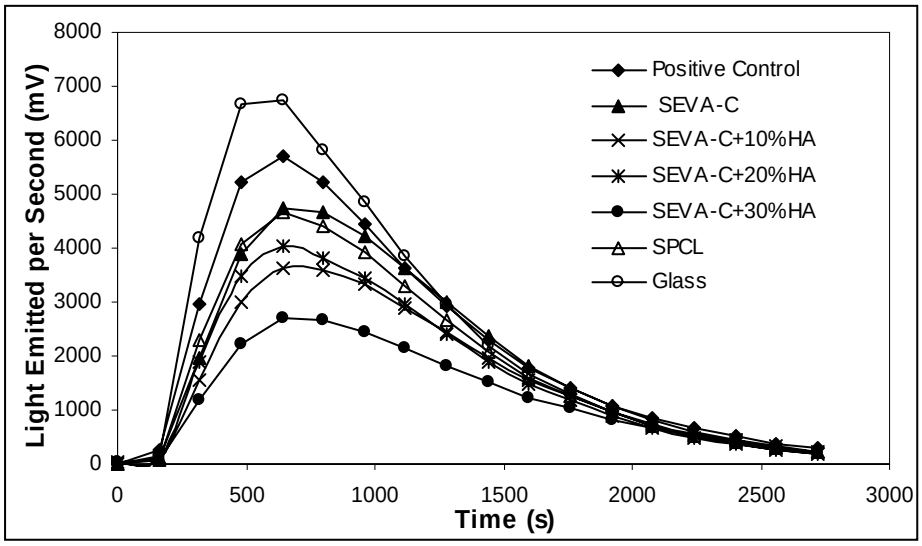

Figure 5.5. Chemiluminescence measurements of the reaction of peroxinitrite with the photoprotein Pholasin® in the presence of two starch-based polymers (SEVA-C and SPCL), respective composites and glass. Positive control corresponds to the polystyrene tube (luminometer cuvette) in the same conditions as the tubes with the materials. The negative control corresponds to the luminometer cuvette without cells and buffer instead. SIN 1, which promotes the simultaneous production of nitric oxide and superoxide and that react between them originating peroxinitrite, was injected to all the cuvettes. Graph represents one experiment, with 2 replicates of each sample.

There is evidence that indicates that secretion of hydrolytic enzymes and production of oxygen metabolites are directly regulated by a dynamic actin filament system through the association of components of NADPH-oxidase, β2-integrins and actin cytoskeletal structures.[27]

In the case of all the materials studied, however, it can be speculated that the reduction of light emitted is the result of the surface properties of the materials on neutrophils adhesion simultaneously with a competition for the photoprotein in solution.

5.4 CONCLUSIONS

Both lysozyme and chemiluminescence assays revealed a low response of the neutrophils when in contact with starch-based polymers and composites.

The hypothesis that the results obtained would be due to an effect on cell adhesion or due to the presence of antioxidant species that would scavenge the reactive oxygen species, considered so harmful for the tissues, was proved, which allows for considering starch-based materials with weak potential to break out an inflammatory response.

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CHAPTER 6

THE EFFECT OF STARCH-BASED BIOMATERIALS ON LEUKOCYTE ADHESION AND ACTIVATION *IN VITRO*

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ABSTRACT

Leukocyte adhesion to biomaterials has long been recognised as a key element to determine their inflammatory potential. Results regarding leukocyte adhesion and activation are contradictory in some aspects of the material's effect in determining these events. It is clear that together with the wettability or hydrophilicity/hydrophobicity, the roughness of a substrate has a major effect on leukocyte adhesion. Both the chemical and physical properties of a material influence the adsorbed proteins layer which in turn determines the adhesion of cells.

In this work polymorphonuclear (PMN) cells and a mixed population of monocytes/macrophages and lymphocytes (mononuclear cells) were cultured separately with a range of starch-based materials and composites with hydroxyapatite (HA). Cell adhesion and differentiation was characterised based on the expression of several adhesion molecules. A combination of both reflected light microscopy and scanning electron microscopy (SEM) was used in order to study the leukocyte responses in terms of morphology. The quantification of the enzyme lactate dehydrogenase (LDH) was used to determine the number of viable cells adhered to the polymers. The expression of adhesion molecules, crucial in the progress of an inflammatory response, was determined by immunocytochemistry.

The work support previous *in vitro* studies with PMN and monocytes/macrophages, which demonstrated that there are several properties of the materials that can influence and determine the biological response. In the present work it was found that more hydrophilic surfaces induced higher PMN adhesion while in the case of monocytes/macrophages and lymphocytes, the opposite was observed.

The results reported herein indicate the low potential of the starch-based biodegradable polymers to induce inflammation especially the HA reinforced composite materials.

It is suggested that the protein layer adsorbed to the surface of the materials immediately after contact with culture medium is the more important factor to determine cell adhesion and activation *in vitro*.

6.1. INTRODUCTION

One of the major stages in the development of biomaterials is the assessment of the biological reactions resulting from their interaction with the living tissues.[1-3] Clinical deployment or application will trigger foreign body reactions that may, according to the severity determine the success or failure of the device.

Cellular recruitment into the interface of tissue and device plays an important role in implant loosening. The cell types that predominate in the implantation site during the different phases of inflammation are lymphocytes, monocytes and macrophages with polymorphonuclear neutrophils (PMN), in acute inflammation or infection[4,5]. These cells constitute appropriate systems to study, *in vitro*, the complex biological reactions of cell-material interactions and the release of chemotactic mediators that *in vivo* will control inflammatory responses.

The mechanisms involved in the development of the inflammatory response are many and rather complex, but the activation of leukocytes leading to the up-regulation of adhesion molecules on the cell surface plays a central role and has been the focus of some recent studies.[6,7] Therefore it is of extreme importance to try to understand the mechanisms of leukocyte adhesion and it's relation with the activation state of the cells. Cells adhere by utilising three major groups of adhesion molecules[8]: integrins, selectins and glycoproteins, for example members of the immunoglobulin superfamily. Cell-cell contacts formed by integrins contribute to activities such as antigen presentation, cytotoxicity, phagocytosis among others.[8,9] Integrins are constitutively expressed on leukocytes, but are only able to form adhesive contacts with other cells following activation to produce structural and affinity changes in the external integrin moieties.[9]

Several adhesion molecules are known to play primordial roles in the inflammatory process, some of them previously used as specific cell-function markers were chosen to be identified in this study. The surface antigen known as lymphocyte function-related antigen (LFA-1), expressed in all leukocytes, is an integrin consisting of an α subunit, also defined as CD11a, and the β_2 subunit, denominated as CD18. The subunit α can vary in the heterodimer, originating to two other important adhesion molecules, the CD11b or Mac-1 and CD11c, both expressed on monocytes/macrophages and granulocytes[10] but not on lymphocytes[11]. CD11a is involved in the adhesion of leukocytes to endothelium during inflammatory reactions and Mac-1 plays a key role in the adherence of both monocytes and neutrophils to vascular endothelium for subsequent extravasation.[12] CD11b/CD18 is also involved in a variety of cell-

cell and cell-substrate interactions such as attachment and phagocytosis of particles coated with C3bi by granulocytes and macrophages.[13]

Integrins have as receptors, specific cell surface molecules belonging to the immunoglobulin (Ig) superfamily, which are expressed on endothelial cells. The β_2 integrins primarily recognise the intracellular adhesion molecule -1 (ICAM-1). In the mediation of cell adhesion, lymphocytes mainly use LFA-1 to interact with ICAM-1 whereas neutrophils appear to use both LFA-1 and Mac-1 to attach to ICAM-1 expressing cells. These are necessary interactions to stop leukocytes rolling along endothelium, enabling migration to the site of injury/inflammation.[12] ICAM-1 binds not only to leukocyte integrins but also to fibrinogen, which may be an important means of recruiting inflammatory cells to places of injury.

Another feature of inflammation involves the expression of major histocompatibility complex (MHC). In particular, during the immune response to pathogens, antigen presenting cells process and present selected foreign peptides through the MHC class I or II on their surface.[13] The capacity of these molecules to avoid interactions between T cells and their accessory cells, has been demonstrated previously[14].

In the presence of biomaterials, however, the typical inflammatory process is different, since materials can determine the extent and duration. The general mechanism of surface dependent cellular responses is believed to be adhesion receptor binding to the surface specific adsorbed protein layer, which activates intracellular signal transduction pathways, resulting in a modification of cell behaviour[15]. Therefore, biomaterial surface physical and chemical characteristics directly or indirectly dictates cell adhesion and activation by determining the types, levels and conformations of adsorbed proteins.[5]

Starch-based materials and composites have been proposed for a large range of biomedical applications.[16-20] These materials have shown promising properties in terms of cytocompatibility[21-23] which leads their evaluation further on to consider their immunogenic potential. The aim of the present study was to investigate the contribution of various types of starch-based materials and composites and respective changes in their chemical and physical properties in leukocyte adhesion and activation, namely in promoting differentiation of different subsets of macrophages in order to demonstrate the effect of these materials in terms of an immunogenic response.

6.2. MATERIALS AND METHODS

6.2.1 Materials

The materials studied were: i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C, Novamont, Italy), ii) SEVA-C reinforced with 10%, 20% and 30% (wt) of hydroxyapatite (HA, Plasma Biotal, UK), iii) a 50/50 (wt %) blend of corn starch and cellulose

acetate (SCA, Novamont, Italy), iv) SCA reinforced with 10%, 20% and 30% (wt) of hydroxyapatite, v) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL, Novamont, Italy) and vi) SPCL reinforced with 10%, 20% and 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

Poly-L-Lactide (PLLA, Purac biochem BV, The Netherlands), being the gold standard for biodegradables in biomedical applications, was used as a biodegradable control material. Borosilicate glass (BDH, England) and polystyrene coverslips (PS, Sarstedt Inc, USA) were used as experimental controls for assays involving neutrophils and monocytes/macrophages separately.

All the materials, both the polymers and the composites were processed into circular samples (∅ 1cm) by injection moulding, under optimised processing conditions and sterilised by ethylene oxide (EtO) in conditions that have been described previously[24].

6.2.2 Neutrophil Isolation

Neutrophils were isolated from fresh heparinised peripheral human blood collected from healthy volunteers. Blood was mixed with a 6% dextran solution, settled and the supernatant layered onto lymphocyte separation medium and centrifuged at 2400 rpm for 25 minutes at room temperature. The pellet was washed once with phosphate buffered saline (PBS) solution without calcium and magnesium and the remaining red blood cells were removed by water lysis. The cell suspension was washed twice with PBS without calcium and magnesium at 2400rpm for 5 minutes at room temperature. Cells were counted using a haemocytometer and kept at 4°C until use.

6.2.3 Mononuclear Isolation (Lymphocytes and Monocytes/Macrophages)

A mixed population of lymphocytes and monocytes/macrophages was isolated from healthy human volunteers. Blood was layered onto lymphocyte separation medium (LymphoSep, ICN Biomedicals, USA) and centrifuged at 2400rpm for 25 minutes at 4°C. Cell suspension was washed twice with PBS at 2400rpm for 5 minutes at 4°C and the final concentration set with Medium 199 (Gibco BRL, USA) supplemented with 1% of antibiotics (Sigma Chemical Co, USA), 10% of foetal calf serum (FCS, Gibco BRL, Life Technologies,USA) and 0.2% of fungizone (Sigma Chemical Co, USA).

6.2.4 Adhesion and Morphological Analysis

After isolation, neutrophils were seeded onto the materials at a concentration of $4x10^4$ cells/ml in 1.5ml of culture medium for ½, 1 and 4 hours and stained with haematoxylin.
The mixed population of lymphocytes and monocytes/macrophages was also cultured in direct contact with the materials for 3, 7 and 14 days, at a concentration of $4x10^4$ cells/ml, in 1ml of the culture medium used to prepare the cell suspension.
After each time period, the cells were rinsed in PBS and fixed with glutaraldehyde 2.5% in PBS for 1 hour at room temperature. After fixation the cells were rinsed with PBS, distilled water and dehydrated in graded ethanol solutions (70%, 90%, and 100%) twice, 15 minutes each and critical point dried. Samples were chromium sputter coated (Emitech K575 X, UK) and observed on a Leo 1550 field emission SEM (Leo, UK).

6.2.5 Lactate Dehydrogenase Quantification

Mononuclear cells were seeded in direct contact with the polymers for 3, 7 and 14 days at a concentration of $5x10^4$ cells/ml, 1.5ml per well. After each time period, materials were transferred to new culture plates together with 500μl of the culture supernatant and all the plates were frozen at -80°C for approximately 60 minutes followed by thawing at 37°C for 60 minutes. This freeze and thaw cycle was repeated 3 times. Supernatant (50μl) of each well was transferred to a new 96-well plate and the lactate dehydrogenase (LDH) kit (Promega, CytoTox96^{TM}) was used to quantify the enzyme. The absorbance of the reaction product was recorded on a multiwell microplate reader at 490nm within 1 hour. A standard curve was prepared with dilutions of an LDH standard versus absorbance readings in order to determine, the LDH Units of each sample. Each sample was tested in triplicate and in 4 separate experiments.

6.2.6 Antibodies

To identify individual leukocyte cell surface molecules the following mouse anti-human monoclonal antibodies were used: CD3 (reacts with ε-chain of the CD3/T-cell antigen receptor) and CD5 as T-lymphocyte markers (Pharmingen, USA), CD11a also known as lymphocyte associated antigen-1 (LFA-1) and expressed by all leukocytes (Pharmingen, USA), CD11b which reacts with Mac-1 and CD11c (Pharmingen, USA) as macrophage and monocyte markers, CD54 (Pharmingen, USA), which reacts with intracellular adhesion molecule-1 (ICAM-1) expressed in activated macrophages, CD68 (Dako A/S, Denmark) a marker for macrophages and HLA-DR antibody (Serotec, UK) which recognizes MHC II antigen present in activated macrophages.

6.2.7 Immunocytochemistry

After each time period materials with cells were washed twice in PBS, fixed with 4% formaldehyde, 2% sucrose solution in water for 30 minutes at room temperature, washed with PBS buffer and stained with the avidin-biotin alkaline phosphatase technique. Materials were exposed to rabbit serum for 30 minutes to reduce nonspecific reactivity, followed by primary antibodies for 1 hour at room temperature. After that time materials were rinsed with PBS for 5 minutes and incubated with biotinylated rabbit anti-mouse IgG antibody (Dako A/S, Denmark) for 1 hour at room temperature. The Avidin and Biotinylated horseradish peroxidase complex (Vector Laboratories Ltd., UK) was added to all materials for 1 hour and the substrate reaction was developed using the Alkaline Phosphatase Substrate Kit (Vector Laboratories Ltd., UK). Each incubation, except the rabbit serum, was followed by one wash with PBS buffer for 5 minutes. Materials were washed and counterstained with haematoxylin and mounted in permanent aqueous mounting medium (Serotec Ltd, UK). Each material had one sample stained as a control replacing the primary antibody with PBS buffer.

6.2.8 Statistical Analysis

LDH data was averaged and the standard deviation is reported as a measure of sample variation. The data was statistically analysed by a one way ANOVA analysis using a Tukey-HDS test.[25] All the materials were compared between themselves and the control. If probability values were less than 0.05 ($p<0.05$), differences observed for the two materials were considered statistically significant.

6.3. RESULTS

6.3.1 Cell Adhesion and Morphology

6.3.1.1 PMN

Neutrophils were cultured in contact with the different materials and their morphology observed by reflected microscopy after haematoxylin staining. The control materials (PLLA as a biodegradable control and glass as the material which induces high PMN activation) showed reduced neutrophil adhesion with a very uniform round morphology. A qualitative microscopic comparison of the amount of cells on the surface of the materials was done. Comparing equivalent times of culture, the blend of starch and cellulose acetate presented higher cell adhesion in contrast with the blend with polycaprolactone which showed a low number of cells on their surfaces. Furthermore, while for SEVA-C, SCA and respective

composites the highest adhesion time was 2 hours of culture, for SPCL and composites it was possible to observe more adherent cells after 4 hours of culture (Fig. 6.1 A, B, C).

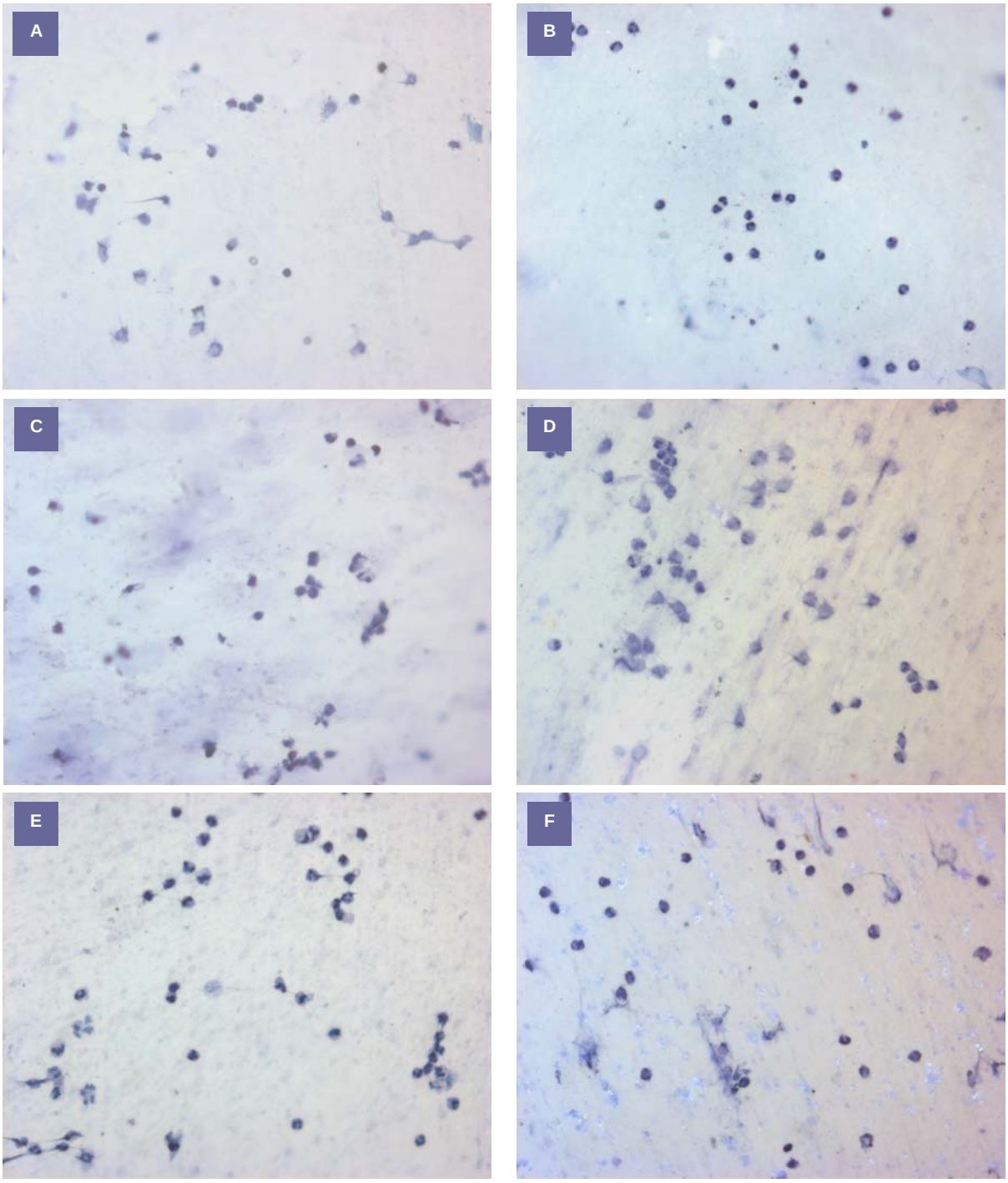

Figure 6.1. PMN cultured on the surface of starch-based polymers and stained with haematoxylin (X20). A) SEVA-C after 2h of culture; B) SPCL after 4h of culture; C) SCA after 2h of culture; D) SEVA-C+10%HA after 2h of culture; E) SPCL+30%HA after 4h of culture; F) SCA+20%HA after 2h of culture.

The presence of ceramic[26] resulted in different affinities, in terms of number of adherent cells, depending on the blend. While starch and ethylene vinyl alcohol composites presented a

tendency for lower numbers of adherent cells, the surface of SCA composites seemed to promote cell adhesion and the reinforcement of SPCL did not show any significant adhesion effect (Fig. 6.1 D, E, F).

The three starch-based biomaterials showed various cell morphologies depending on the type of blend, but morphology also changed in the presence of HA. Some neutrophils on the surface of SEVA-C adopted a spindly, elongated morphology suggestive of motility (Fig. 6.1A). However, in the presence of ceramic at 10% HA, the majority of the cells spread extensively (Fig. 6.1D) and in the presence of 20 and 30% of HA, cells remained quite round.

For the starch with cellulose acetate materials, variations in the morphology of adherent cells were only observed in the presence of the composite with 20% ceramic (Fig. 6.1F) where it was possible to see some individual cells with extended philopodia. The unreinforced polymer and the composites with 10 and 30% of HA showed that neutrophils on these surfaces assumed a round morphology although with some spreading, having the tendency to agglomerate forming clusters.

For the starch-based blends with polycaprolactone there was only a percentage of HA that showed differences in neutrophil adhesion. For SPCL and it's composites with 10 and 20% of HA, cells presented a round morphology comparable to isolated cells (Fig. 6.1B) while in the composite with 30% of HA, adherent cells were bridging to other cells forming structure with a chain-like shape (Fig. 6.1E). It should be noted that these differences were observed after 4 hours of culture and after 2 hours they looked alike on the surface of any of the starch-polycaprolactone polymers or composite materials.

6.3.1.2 Monocytes/macrophages and Lymphocytes

The SEM observation of the mixed mononuclear population of monocytes/macrophages and lymphocytes cultured in direct contact with the materials in the study demonstrated that, in fact, the different types of cells present in culture were adhered to the different surfaces. Lymphocytes were identified by being much smaller in size than monocytes/macrophages and presented a very round morphology. The morphology of monocytes/macrophages varied depending on the material. Non activated monocytes were round cells without philopodia and with many microvilli on the cell membrane surface. PLLA and PS were used as control materials; these materials presented a smooth surface, and it can be seen in figure 6.2A and 2B that cells showed cytoplasm extensions towards the materials surface. SCA and it's composites were found to be the surfaces where monocytes/macrophages spread out (Fig. 6.2E). The majority of the cells displayed long cytoplasmic extensions especially in the case of the unreinforced polymer. On the SCA composites, cells presented fine philopodia which seemed to be looking for the HA particles.

In contrast to starch-cellulose acetate materials, cells adherent to SPCL and it's composites were found to maintain a round morphology comparable to non activated cells (Figure 6.2C). Neither the presence of ceramic nor its amount affected cell adhesion.

Comparing all the materials, SEVA-C and its composites resulted in an intermediate mode of adhesion. It was possible to observe a considerable amount of monocytes/macrophages spread on these surfaces, although not showing significant philopodia. Cells were flattened on the surfaces using all of their cytoplasm instead of fine cell extensions (Fig. 6.2D).

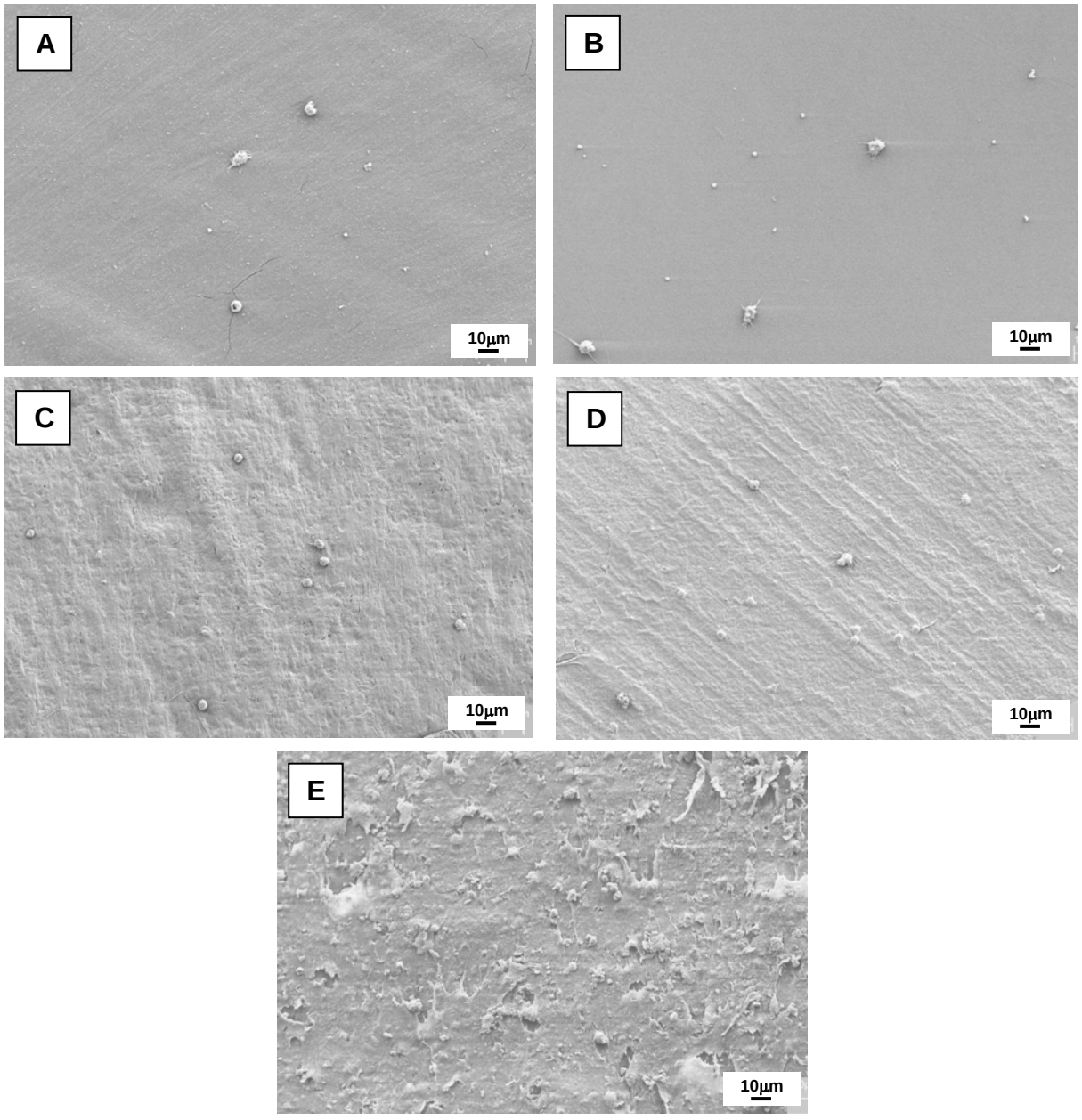

Figure 6.2. Scanning electron micrograph showing a mixed population of monocytes/macrophages and lymphocytes cultured on the surface of different polymers for 7 days. A) PLLA; B) PS; C) SPCL; D) SEVA-C; E) SCA+30%HA.

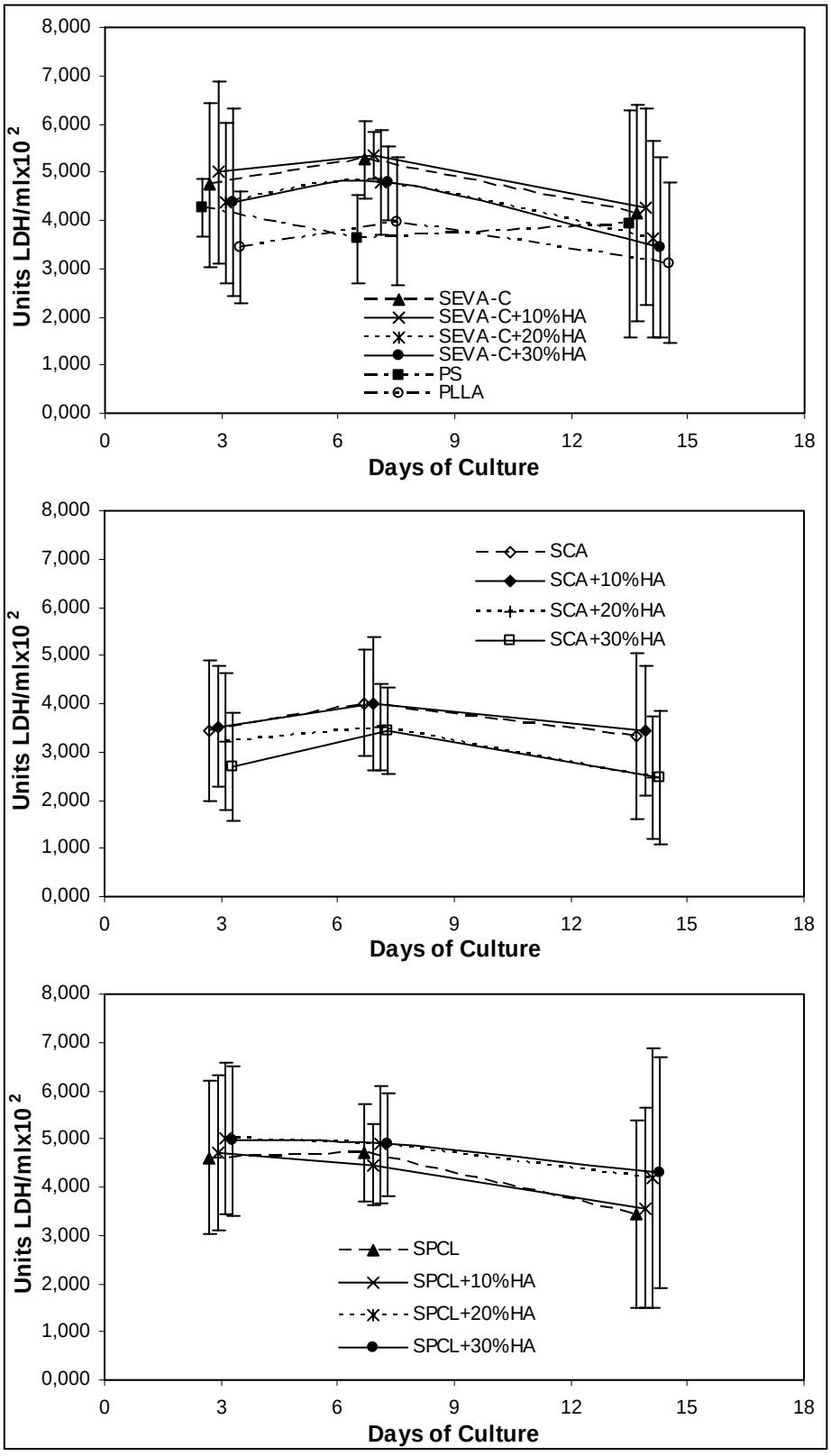

Figure 6.3. Concentration of lactate dehydrogenase (LDH) in the cell cytoplasm, from a mixed population of monocytes/macrophages and lymphocytes, adhered to the polymers and composites after 3, 7 and 14 days of culture.

The quantification of cells by LDH determined that SEVA-C and it's composites were the surfaces that promoted more monocyte/macrophage and lymphocyte adhesion (Fig. 6.3A) and that for SCA and it's composites less cells adhered (Fig. 6.3B).
Considering all blends, the results for unreinforced polymers and composites with 10% of HA were found to be similar as well as the results between the composites with 20 and 30% of ceramic. Thus, for each blend and considering the amount of adherent cells it was possible to divide the materials in two groups. For the materials based on starch with ethylene vinyl alcohol and cellulose acetate (Fig. 6.3 A, B) higher amounts of HA results in fewer adherent cells, while in the case of SPCL materials the opposite was verified (Fig. 6.3C).
The number of cells on the surface of SEVA-C and SCA materials was also found to increase from 3 to 7 days of culture and decrease from that time on, while in the case of materials of starch with polycaprolactone the number of cells tends to decrease from 3 days until the end of the experiment.
The number of cells on the surface of the control materials was equivalent to SCA and its composite with 10% of HA (Fig. 6.3 A, B).

6.3.2 Immunocytochemistry

Although *in vitro* conditions may influence the functional behaviour of cells, this study was performed in order to correlate morphological observations and the functional activity of monocytes/macrophages and lymphocytes, *in vitro*, in the presence of novel potential biomaterials. Image analysis was attempted in order to quantify the number of cells expressing each antigen, however it was not possible to apply that technique since many details, apart from the cells, on the surface of starch-based materials were also stained which resulted in significant errors in the numbers obtained.
The surface markers on macrophages have shown modulated expression because of contact with materials. Our results highlighted the presence of distinct functional subsets of macrophages. These subsets exhibit morphological, immunophenotypic and functional differences depending on the polymer substrate. From SEM analysis, lymphocytes were quite easy to identify presenting a very round shape. This was confirmed from positive staining using CD3 (Fig. 6.4A) and CD5 antigens. Adherent lymphocytes on the surface of all the tested materials demonstrated that the expression of CD3 and CD5 was affected by the type of material and for some materials, also by the time of culture. For the surfaces of PS and SPCL the number of CD3 positive lymphocytes seemed to decrease with time. In the case of the other materials the amount of cells expressing CD3 did not change significantly for longer periods of culture. Furthermore, the amount of CD5 positively stained cells did not significantly vary with increasing time. Moreover, the composites presented a similar number

of cells expressing both CD3 and CD5 antigens, compared with the unreinforced polymer. Lymphocytes seemed to be present in higher numbers on the surface of PS after 3 days of culture, decreasing to fairly similar amounts to the other materials after 7 days. It should be stressed that these statements are based on microscopical observations; the total number of cells on the surface of the materials does not vary as demonstrated by the statistical analysis of the LDH quantification.

The identification of the CD11/CD18 integrins expressed by leukocytes adhered to the surface of the different polymers by immunostaining with anti- LFA-1, Mac-1 and CD11c antibodies, confirmed the existence of different sub-populations of cells. It was possible to identify CD11c positive macrophages as a sub-population of cells which spread according to substrata (Fig. 6.4D). Two other sub-sets of macrophages showing different levels of attachment/spreading were found to be CD11b positive (Fig. 6.4C). Cells stained with anti-CD11a antibody confirmed the attachment of different cell types, lymphocytes and monocytes and the sub-populations of macrophages (Fig. 6.4B).

The culture time influenced the number of cells expressing CD11/CD18 molecules, although the numbers of mature macrophages (CD68 positive cells, Figure 6.4E) seemed to increase on PS and SPCL for longer culture periods. In particular, In the presence of PS and SEVA-C the number of CD11b positive cells appear to increased with the time of culture. An opposite tendency was presented by cells expressing CD11c after adherence to PLLA which seem to be in lower amounts for longer times of culture. Furthermore for increasing time of culture, more cells expressing LFA-1 and CD11c, were found adherent to SEVA-C and SPCL respectively.

The different materials in the study also affected the monocyte/macrophage phenotype. The number of macrophages (positive cells for CD11c) adherent to the surface of the PS control is much higher than on any of the other polymers, which were found to induce CD11c expression at similar levels. PS, SCA and SPCL were also found to up-regulate the expression of Mac-1 when compared to SEVA-C and PLLA.

Another interesting result involves the presence of HA. The presence of the ceramic did not seem to affect CD11c expression in the cultured cells when comparing to unreinforced materials. However, although increasing amounts of HA did not seem to induce significant differences in the expression of CD11/CD18 adhesion molecules, it was possible to observe that HA down-regulates Mac-1 expression on cellulose acetate and polycaprolactone containing composites and induces spreading on cells adherent to SCA composites and expressing LFA-1. Furthermore, CD68 antigen was found to be down-regulated in the presence of all the composites when compared to the polymers without ceramic.

Considering the activation state of the cells cultured in contact with starch-based materials it was possible to observe that in fact, macrophages expressing CD54 (ICAM-1), showed a

particular morphology when compared with other cells stained with different antibodies (Fig. 6.4F). Cells were shown to be well spread on the surface of the polymers and in lower numbers on SCA and PLLA. In addition, SEVA-C and SPCL composites induced a down regulation in ICAM-1 expression.

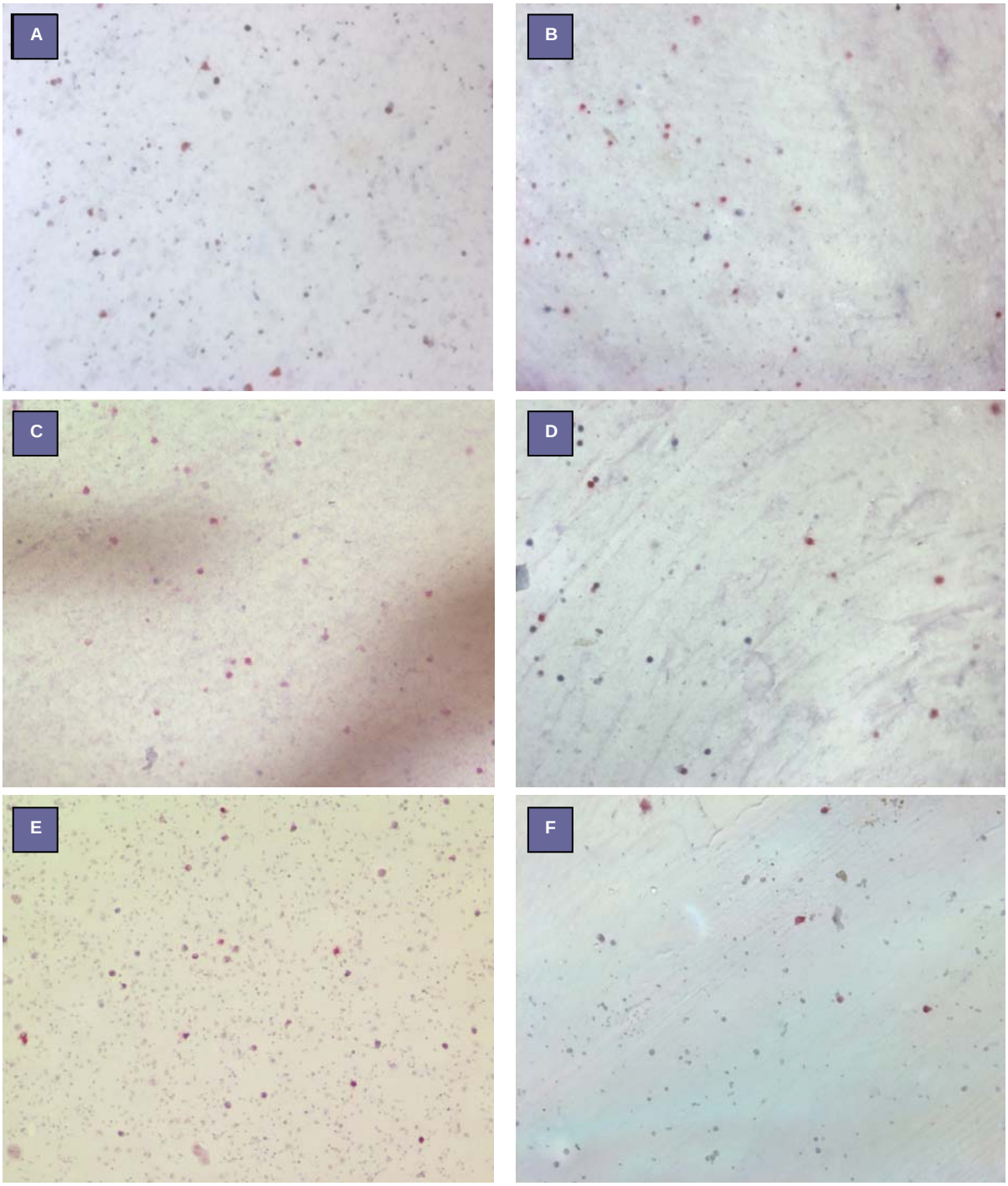

Figure 6.4. Immunostaining of mixed cell populations of monocytes/macrophages and lymphocytes cultured for 3 days (D), 7 days (A,B,F) and 14 days (C,E) on: A) SEVA-C+20%HA, B) SCA+20%HA; C) SEVA-C, D) SEVA-C+10%HA, E) PS, F) PLLA. Cells were stained using CD3 (A), CD11a (B), CD11b (C), CD11c (D), CD68 (E) and CD54 (F) antibodies (red cells) and counterstained with haematoxylin (purple cells). Magnification (X10)

This study also demonstrates a significant difference in antigen-presenting phenotype in adherent cells. SEVA-C, PLLA and PS showed fewer cells expressing the HLA-DR antigen, however for longer culture times, the number of cells presenting antigen decreased for SEVA-C and increased for PLLA as well as for SCA.

6.4. DISCUSSION

Polymorphonuclear neutrophils are found at the surface of the materials 10 minutes after blood exposure and the cells become activated after 30 minutes to an extent that depends on the implanted device[27]. These cells may become activated either directly through adhesion receptors, or indirectly via platelet-derived mediators. However, the adhesion-mediated mechanisms of PMN activation are not well understood and several works have shown conflicting results. Some studies presented evidence that cell adhesion has an important role in supporting or preventing neutrophil apoptosis.[28,29] In addition, interactions between neutrophils and a biomaterial surface have been suggested to cause premature activation of cells which causes a long-term down-regulation of neutrophil function on biomaterial surfaces *in vitro*[30]. Other researchers have shown reduced oxidative responses from adherent PMNs on different surfaces.[31,32] A previous study with starch-based materials also revealed that the production of degradative enzymes and reactive oxygen species was reduced when in contact with those materials.[32] Based on this it could be suggested that SPCL and composites would not promote immediate neutrophil adhesion, which would result in a more intense short-term activation that would be reduced after adhesion.

It is defended[33-35] that *in vivo* and *in vitro* (in the presence of serum) biomaterials instantly become associated with plasma and/or matrix proteins, which have the potential to modify the interactive effects of materials and cells. Proteins may however act in different ways. Some studies[33,35] demonstrated that some proteins enhance neutrophil activation, but other research showed that they can inhibit PMN resulting in a diminished inflammation[33,34]. The amount of protein pre-adsorbed on a surface can also affect cell adhesion by masking the surface properties of the underlying substrate and thus minimising the non-specific interactions.[36] Not only the type of proteins or their concentration, but also the way they adsorb to the materials is determinant for cell adhesion. This dependence ultimately relies on the physical and chemical nature of the surfaces. In fact, oxidised surfaces were shown to stimulate granulocyte activation [37]. Moreover, PMN were observed to use different receptors to adhere onto hydrophilic and hydrophobic surfaces [38].

The present *in vitro* study was performed in the presence of serum, a complex system of different proteins which adsorb to the surface of the different materials and consequently modulate cell adhesion, depending on the material properties. Previous studies with starch-

based blends showed that the three materials present a different surface in physical[16] and chemical terms[39]. In fact the SCA polymer, the material with higher number of adhered PMNs, has a higher oxygen content on it's surface [39].

It can also be speculated that the presence of HA influences neutrophil adhesion in highly (SCA) and moderate (SEVA-C) hydrophilic surfaces in different ways respectively inducing and reducing the number of adherent cells probably due to their wettability. The incorporation of ceramic reduces hydrophilicity which will reach a more favourable value in the case of SCA, but transforming SEVA-C to a less adherent surface for neutrophils.

The neutrophil spreading and migration over a surface requires rearrangements of the actin cytoskeleton, which together with focal contacts regulate the dynamic interactions between β2-integrins and extracellular matrix proteins. A correlation between cell spreading, pseudopodia formation and activation state was previously suggested by Wettero et al. [40] Other authors[41] reported similar results although suggesting a dependence of cell adhesion for oxygen radical production.

Thus considering the predisposition of each material in the study to attract PMNs, the morphology cells assume when adherent and previously reported results on reactive oxygen production and degradative enzyme release in the presence of starch-based polymers, it can be suggested that starch-based materials that promote more neutrophil adhesion tend to inactivate PMN.

Contrarily to PMNs, macrophage lineage is known to be heterogeneous, and considerable variability in cell morphology has been noted after activations for example by cytokines or other chemical moderators such as lipopolysaccharides (LPS).[42,43] Under the influence of cytokines, macrophages show various features of activation. Morphologically, the cell increases in size. The number of cytoplasmic granules increases and the plasma membrane becomes more ruffled. Phagocytic activity is also another characteristic that activated macrophages may exhibit upon attachment to a surface. Activated macrophages spread more rapidly and extensively than resting macrophages[44].Therefore, cellular adhesion and spreading on material surfaces appears to be an attempt by the macrophage to phagocytose the implanted device.

Some studies[45,46] have focused on the capacity of macrophages to degrade ceramic coatings or to phagocytose HA particles through the extension of cytoplasmic pseudopodia around particles until completely encapsulated.

The lower numbers of cells in the presence of SEVA-C and SCA composites and their tendency to decrease with increasing percentages of HA might suggest preferential monocyte/macrophage adhesion to ceramic particles when comparing the surface of the polymers. It may be hypothesised that polymer/HA interfaces were more susceptible to

degradation inducing the release of HA particles which in turn attract the phagocytes therefore decreasing the number of cells on the surface of the materials.

When in contact with starch-based materials and composites, leukocytes were not observed bridging and no apparent macrophage fusion into giant cells was determined, although macrophage aggregation, fusion and formation of foreign body giant cells (FBGC) on the surface of biomaterials has been previously observed *in vitro*[47], and also at the interface between tissue and retrieved smooth surface implants[48]. Several studies[49,50] have shown that long-term macrophage densities and FBGC formation can be greatly influenced by substrate chemistry.

There is some controversy about the factors that affect and how they affect leukocyte adhesion to biomaterials surfaces. Wettability and morphology of the materials were shown to play an important role; porous and more hydrophilic surfaces were found to have more adhered monocytes[51,52] but some authors[53-55] have concluded that macrophages preferentially accumulate on rough and hydrophobic surfaces *in vitro*. Considering each one of these variables independently, we could say that from our study monocyte/macrophages and lymphocytes adhere in similar amounts to more hydrophobic (SPCL) and to moderately hydrophilic (SEVA-C) surfaces and do not adhere preferentially to rougher substrates since SCA is the polymer with the most irregular surface.

However, not only those properties of the surfaces of biomaterials are known to affect monocyte/macrophage adhesion and activation. Since these cells bear a negative charge due to their lipoprotein membrane structure therefore inhibiting cell adhesion to negatively charged surfaces, it was suggested that electrostatic forces may influence leukocyte adhesion. Furthermore Anderson et al[52] identified the potential of surface chemistry-dependent conformational alterations, which may occur in proteins adsorbed to surfaces. Specific fibronectin fragments are potent chemoattractants for human blood monocytes, while the intact molecule is not chemotactically active[56]. On the other hand, surfaces that preferentially adsorb vitronectin from serum containing medium are favourable for macrophage adhesion[57,58]. A previous study with starch-based materials showed that from human serum, vitronectin adsorbs onto starch ethylene vinyl alcohol based materials and that more monocytes/macrophages and lymphocytes were present at short times of culture on these surfaces.[59] In the present study, proteins play a crucial role, since all the experiments were performed in culture medium containing serum, which provides a rich assortment of adsorbed ligands for adhesion receptors. The results are in accordance with that study since SEVA-C and composites were the materials that showed higher cell adhesion, which suggests a possible mediation of vitronectin in monocyte/macrophage and lymphocyte adhesion to that starch-based blend.

For leukocytes, cell-cell-contacts formed by integrins contribute to several activities such as antigen presentation, cytotoxicity and phagocytosis.[9] However, in the presence of biomaterials, leukocyte integrins undergo an activation process during which changes in affinity (conformation) and avidity (post-receptor occupancy) can up-regulate ligand-binding activity. Conformationally sensitive integrin binding with specific adsorbed peptide sequences is believed to provide anchorage and stimulate signal transduction pathways of adherent cells in a surface dependent manner.[60]

Mac-1 (CD11b/CD18) plays a key role in the adherence of both monocytes and neutrophils to vascular endothelium and has been implicated in the evaluation of cell activation.[61-63] Following stimulation CD11b/CD18 is rapidly mobilised from intracellular stores to the cell membrane and although an increase in receptor expression of CD11b/CD18 can result in increased cell adhesion it was previously suggested that not all newly recruited receptors are believed to be functional.[63] Instead further modifications are needed in order to render the receptors functionally competent thus, the expression of CD11b/CD18 cannot fully predict the degree of cell activation.[61,62]

CD11b/CD18 expressing cells are also known to be involved in the phagocytosis. Allying a hypothetical activated state of the CD11b/CD18 positively stained cells with their phagocytic role it might be possible to identify, from the cells adherent to the surface of the materials in study, those which are in fact activated. The sub-type of monocytes/macrophages expressing CD11b/CD18 with a larger spread morphology characteristic of cells which are involved in the phagocytosis process, can be presumed to correspond to those activated cells.

ICAM-1 is part of the immunoglobulin superfamily of adhesion molecules, found to be up-regulated by inflammatory regulators and is used *in vitro* as a marker of activation.[44,64] Bernatchez et al[44] also suggested that ICAM-1 expression at the surface of the cell may be linked more with the extent of cell spreading than to the concentration of soluble inducers. Therefore, the materials which presented lower number of cells expressing ICAM-1 molecules (SCA and PLLA) are expected to induce less short-term inflammation.

Probably a little unexpected, since it would be natural the maturation/activation of monocytes/macrophages in an *in vitro* system where cells are exposed to foreign materials, was the fact that the expression of ICAM-1 did not seem to be affected by the time of culture. The presence of HA down-regulates the maturation of monocytes into macrophages that some composites down-regulates the expression of ICAM-1 molecules together with the expression of CD11b/CD18 integrins.

Polymers can induce a specific immune response in two ways mainly; materials can release various products by interacting with the surrounding tissue and these products can bind adequate tissue carriers and become antigenic or self-proteins are altered, which can be

endocytosed and presented by antigen presenting cells (APCs) to T cells.[65,66] The results for MHCII identification could be explained by considering that SCA is the material that takes up more water and consequently degrades more rapidly. It is possible that the surface changes not only from the materials but also in terms of adsorbed proteins, with the time of culture resulting in increased activity from antigen-presenting cells. The amount of activated macrophages (CD54 positive) was found to be lower in the presence of SCA, which was not in agreement with the results for antigen-presenting cells identification.

Although the protein layer adsorbed to the surface of starch-based biomaterials varied according to the synthetic component of the blend[59], it is not only the type of proteins which are believed to determine the cellular response. It can be speculated that in fact the surfaces themselves induce different conformations and affinities in the adsorbed proteins, which result in diverse signalling mechanisms and consequently in varied cell adhesion molecules expression.

6.5 CONCLUSIONS

An in vitro model was established simulating aspects of the in vivo inflammatory response. The aim was to evaluate individual and collective cellular effects resulting from the interaction of the different populations of inflammatory cells with starch based biodegradable biomaterials.

The inflammatory response to biomaterials was demonstrated to be a very complex process, certainly influenced by the chemical and physical properties of the materials. These factors did not necessarily act independently and also affected the diverse components of the biological system in different ways.

While SCA promoted higher PMN adhesion and lower activation, the number of cells from a mixed population of monocytes/macrophages and lymphocytes was found to be lower on that material also showing a reduced amount of activated macrophages.

In addition, the hydroxyapatite reinforcement induced changes in cell behaviour for some materials but not for others. However, HA generally showed reduced monocytes/macrophage adhesion and less potential to activate the cells.

Comparing the control materials, there was no significant difference between the biodegradable materials; that is between starch-based and PLLA biomaterials. It was possible to verify that PS was from the tested polymers the one that showed the greatest inflammatory potential.

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CHAPTER 7

CYTOKINE SECRETION FROM MONONUCLEAR CELLS CULTURED *IN VITRO* WITH STARCH-BASED POLYMERS AND PLLA

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ABSTRACT

The cytokine network is one of the major controlling systems of the inflammatory process, driving the magnitude and duration of the host response against invading microorganisms, foreign materials or altered internal stimuli. Pro and anti-inflammatory cytokines were quantified after *in vitro* culture of a mixed population of monocytes/macrophages and lymphocytes with biodegradable polymers. Different blends of starch-based polymers and their composites filled with hydroxyapatite were studied and compared with Poly-L-Lactide (PLLA). Interleukin-1 beta (IL1-β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) were investigated as the markers of immunological reactivity as they are known to act at the early stages of injury/invasion. Interferon-gamma (IFN-γ), recognised as a pro-inflammatory cytokine, although not present during early responses was also investigated. Contrarily, interleukin-4 (IL-4) derived from T lymphocytes, was investigated as it is an immunoregulator that counteracts some aspects of inflammation. T lymphocyte activation was also determined by quantifying interleukin-2 (IL-2).

The results support the hypothesis that different biodegradable polymers can affect mononuclear cell activation and the production of several cytokines associated with the inflammatory process. No IL-2 or IFN-γ was found in the culture supernatants after 3, 7 and 14 days in the presence of any of the materials. IL-6 was detected in the highest amounts, for all the conditions, followed by TNF-α. IL-1β was produced in very low amounts, being undetectable with some of the starch-based materials. IL-4 was the only cytokine that did not demonstrate any significant difference within this group of materials.

Starch-based polymers and composites induced lower production of pro-inflammatory cytokines in comparison to PLLA.

7.1. INTRODUCTION

It is now recognised that the failure of implanted medical devices can be associated with several inflammatory and infectious processes, with the overproduction of cytokines by persistent leukocytes potentially being a significant inherent factor in these.[1-3] Adherent and activated mononuclear cells, monocytes/macrophages and T lymphocytes produce inflammatory cytokines and chemokines that recruit other inflammatory cells to the site of implantation.[1,4] Furthermore, cytokines may stimulate non-inflammatory cells to secrete enzymes and cytokines that will compromise the success of the implant by altering cell phenotype and function.[5,6]

Mononuclear cells comprising monocytes and lymphocytes were cultured *in vitro* to determine the effect biomaterial surfaces had on the production and release of IL1-β, IL-6, TNF-α, IL-2, IL-4 and IFN-γ, which was analysed by enzyme linked immunosorbent assay (ELISA). The cytokines were selected considering their established roles in influencing the foreign body response to implanted materials by controlling local inflammation, cellular activation and chemotaxis[7,8]. It is recognised that anti-inflammatory cytokines can also be present to act to reduce inflammation[8], balancing the effects of pro-inflammatory cytokines and maintaining homeostasis. IL-4 was investigated as an important anti-inflammatory cytokine.

The *in vitro* model was composed of different cell types, which produce specific cytokines. IL-1β although produced by many different cell types including neutrophils, natural killer cells, B and T-lymphocytes, is produced in great abundance by blood monocytes and tissue macrophages.[9]

Mononuclear phagocytes also produce IL-6, one of the earliest cytokines of the inflammatory process and a major inducer of acute phase proteins.[10,11]

Lymphocytes, present in the mononuclear population cultured in direct contact with the materials, also produce IL-6 and IL1-β. Nonetheless, IL-2[12], IL-4[13] and IFN-γ[14,15] are specifically lymphocyte derived cytokines and their quantification can help to determine the potential roles of lymphocytes in direct contact with materials.

TNF-α is produced in large amounts by activated mononuclear phagocytes, macrophages, and lymphocytes[16,17] and for that reason represents a valuable activation marker.

Besides the induced level of stimulation of each population of cells, the model enabled the analysis of interactions between the cytokines themselves. IL-1β is known to have multiple roles in the regulation of normal tissue repair and chronic inflammation. It stimulates the production of acute phase-reactant proteins[11] and has the ability to activate wound healing cells (fibroblasts), lymphocytes and monocytes[9,18]. IL-6 is a cytokine with pleiotropic activities produced by a variety of cells including fibroblasts, endothelial cells, mononuclear

phagocytes, neutrophils, hepatocytes and T and B lymphocytes.[19] This cytokine can down regulate fibroblast and endothelial cell growth and alternatively it can promote the growth and differentiation of monocytes and lymphocytes[19] . The lymphocyte-derived cytokines have significant influence in the monocyte/macrophage population synergising and/or reducing the effect of the cytokines produced by those cells. In particular, in response to IL-2, macrophages will synthesise IL-1 that further activates T cells to produce more IL-2 to activate more macrophages in a feed-back cycle.[20] IL-4, secreted predominantly by T helper 2 (Th-2) lymphocytes[13], inhibits production of IL-1 and TNF-α, by activated monocytes[21]. IL-4 has also been shown to promote monocyte/macrophage fusion to form foreign body giant cells (FBGC).[22] IFN-γ exerts important activities on both monocyte/macrophage and lymphocytes, which generally results in macrophage activation and T cell differentiation towards a Th-1 (T helper 1) type of immune response[23].

In this work the potential of starch-based materials, previously proposed for a wide range of biomedical applications[24-27], to induce cytokine production by mononuclear cells *in vitro* was investigated and compared to PLLA.

7.2. MATERIALS AND METHODS

7.2.1 Materials

The materials studied were: i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C, Novamont, Italy), ii) SEVA-C reinforced with 10%, 20% and 30% (wt) of hydroxyapatite (HA, Plasma Biotal, UK), iii) a 50/50 (wt %) blend of corn starch and cellulose acetate (SCA, Novamont, Italy), iv) SCA reinforced with 10%, 20% and 30% (wt) of hydroxyapatite, v) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL, Novamont, Italy) and vi) SPCL reinforced with 10%, 20% and 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

Poly-L-Lactide (PLLA, Purac Biochem BV, The Netherlands), being the gold standard for biodegradables in biomedical applications, was used as a biodegradable control material and Polystyrene (PS) coverslips (Sarstedt, UK) as a reference material.

All the materials were processed into circular samples ($\varnothing$ 1cm) by injection moulding under optimised processing conditions.

7.2.2 Mononuclear Cell Isolation and Culture

A mixed population of lymphocytes and monocytes/macrophages was isolated from healthy volunteers. Blood was layered onto lymphocyte separation medium (LymphoSep, ICN

Biomedicals, USA) and centrifuged at 400g for 25 minutes at 4°C. The cell suspension was washed twice with PBS at 400g for 5 minutes at 4°C and the final cell concentration controlled and taken into cell culture conditions with Medium 199 (Gibco BRL, USA) supplemented with 1% of antibiotics (Sigma Chemical Co, USA), 10% of foetal calf serum (FCS, Gibco BRL, Life Technologies,USA) and 0.2% of fungizone (Sigma Chemical Co, USA).

Cells were seeded in direct contact with the polymers for 3, 7 and 14 days at a concentration of $5x10^4$ cells/ml, 1ml per well.

7.2.3 Cytokine Quantification

After each time period, plates were centrifuged in order to avoid cells in suspension and 200μl of the supernatant of each well was transferred to a new 96-well plate and kept at -80°C, previously shown to be unaffected by up to three freeze thaw cycles[28], for analysis using ELISA. The following cytokines were quantified by enzyme immunoassay. Human Interferon-gamma (IFN-γ) with a minimal detectable dose of <4pg/ml, Human Interleukin-2 (IL-2) with a minimal detectable dose of <5.1 pg/ml, Human Interleukin-4 (IL-4) with a minimal detectable dose of <2.0 pg/ml, Human Interleukin-6 (IL-6) with a minimal detectable dose of <2 pg/ml, Human Interleukin-1 Beta (IL-1β) with a minimal detectable dose of 1 pg/ml, and Human Tumour Necrosis Factor Alpha (TNF-α) with a minimal detectable dose of 1.7 pg/ml. (All ELISA kits obtained from Biosource International, Inc, USA)

For each cytokine all samples were tested in duplicate on each plate and repeated at least 3 times in independent experiments except for IFN-γ and IL-2, which were found to be undetectable after 2 experiments and stopped. ELISA plates were read using a LUCY 1 luminometer plate reader at wavelength 450nm (Bio-Stat, Biochem Immunosystems, Italy). For each measurement a standard concentration curve was generated according to the indication of the assay kit and used to calculate the concentration of released cytokine.

7.2.4 Statistical Analysis

The data was statistically analysed using a one way ANOVA analysis using a Tukey-HDS post hoc test[29]. The results were compared for all the materials at the different times of culture, and between themselves at each time of culture. If probability values were less than 0.05 ($p<0.05$), differences observed were considered to be statistically significant.

7.3. RESULTS

Cytokine production is summarised in table 7.1. IL-6 was detected in the highest amounts, for all the conditions, followed by TNF-α. IL-1β was produced in lower amounts and was undetectable with some of the starch-based materials. No IL-2 or IFN-γ was produced at any of the tested times of culture in the presence of any of the materials.

There were differences in the amount of released cytokines with respect to culture time, the amount decreasing with increasing culture time. In a previous study[30], the same experimental conditions were used for the quantification of the intracellular LDH of cells adherent to the surface of the materials. That data showed that the number of adherent cells varied according to the material, although not statistically significant. The results demonstrated that the cytokine production was affected by the material and not by the adherent cell number.

Table 7.1: Cytokine production at 3, 7 and 14 day Time Periods

	IL-4			IL-6			IL-1β			TNF-α			IFN-γ			IL-2		
Time Culture	3	7	14	3	7	14	3	7	14	3	7	14	3	7	14	3	7	14
Material																		
SEVA-C	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-
SEVA-C+10%HA	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
SEVA-C+20%HA	+	+	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-
SEVA-C+30%HA	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
SPCL	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
SPCL+10%HA	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-
SPCL+20%HA	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
SPCL+30%HA	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-
SCA	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
SCA+10%HA	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
SCA+20%HA	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-
SCA+30%HA	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
PS	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
PLLA	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-

7.3.1 IL-4

The amount of IL-4 detected varied between 16 and 29 pg/ml. No significant differences were observed except in the case of SCA+20%HA (Fig. 7.1c). The levels of IL-4 tended to increase with the culture period in the presence of composites, reaching values after 7 and 14 days of culture which were statistically different from those obtained for 3 days of culture. There was no correlation between the IL-4 released and the different starch-based blends or with the percentage of HA that was reinforcing each of them.

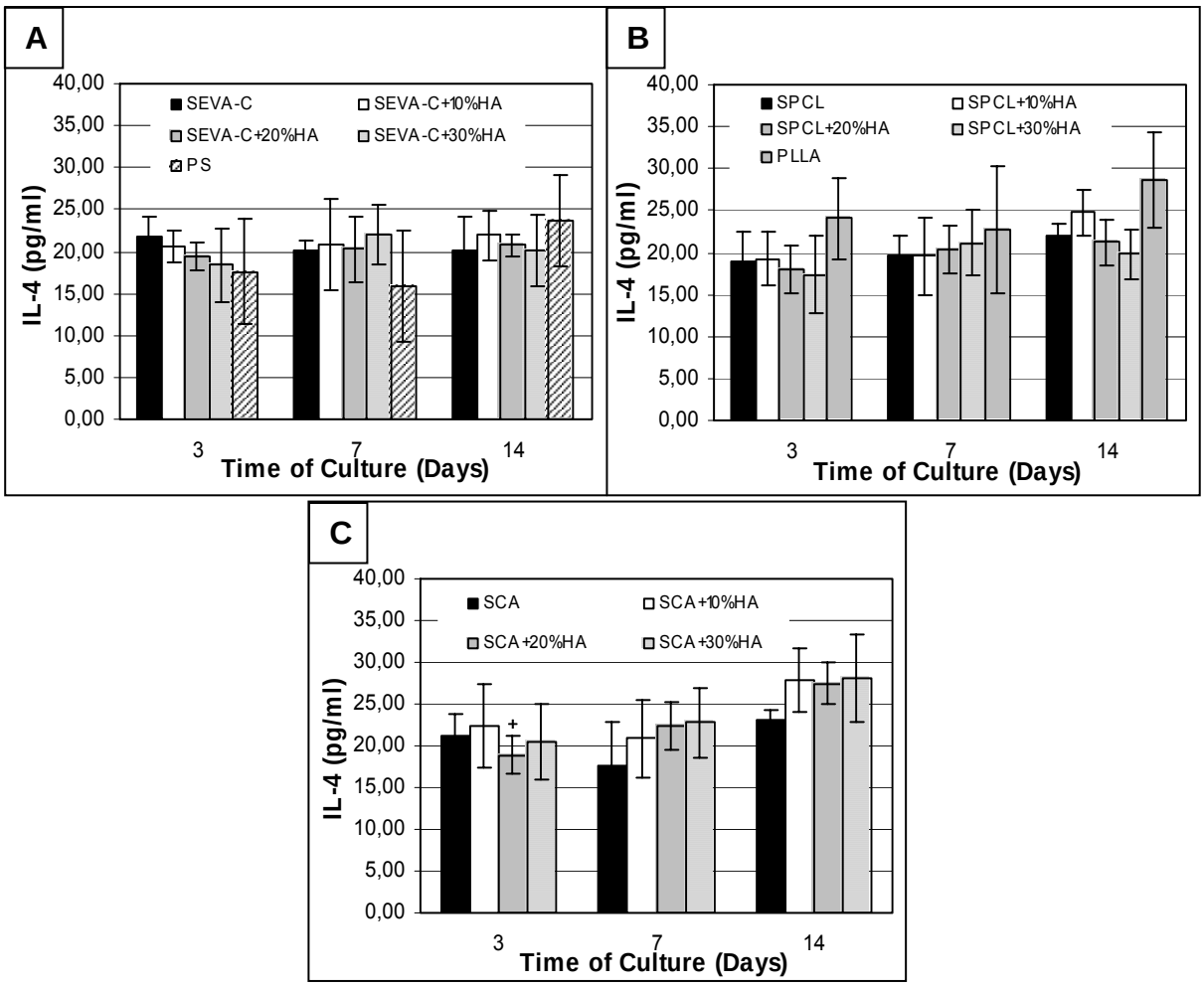

Figure 7.1. IL-4 release from monocytes/macrophages and lymphocytes when in culture with (a) SEVA-C and respective composites with HA and PS, (b) SPCL and respective composites with HA and PLLA, (c) SCA and respective composites with HA, for 3, 7 and 14 days. Data represents mean ± standard deviation, n≥3. $^{+}$ Indicates significant difference with 7 and 14 days of culture.

7.3.2 IL-6

In the case of IL-6, the results obtained were complex and influenced by the type of material and the presence of ceramic reinforcement. It was possible to observe that the amount of IL-6 released decreased with the time of culture for most of the materials although these differences were not statistically significant.

IL-6 was the cytokine detected in highest quantity in the harvested supernatants and varied between about 1200 and 70 pg/ml. PLLA and PS were the two materials which induced the highest IL-6 release. The results obtained in the presence of SEVA-C and composites (Fig. 7.2a) and in the presence of SCA and composites (Fig. 7.2c), after 3, 7 or 14 days of culture were found to be statistically different from those obtained for these two control materials, except SCA after 7 days ($p<0.05$ only when compared with PS). For SPCL and composites (Fig. 7.2b), these materials induced a release of IL-6 similar to that of PS after 3 days (except

SPCL+30%HA) and comparable with PLLA after 7 days. After 14 days, the levels of IL-6 induced by SPCL and composites reached lower values, and these were significantly different from PS and PLLA and therefore closer to those obtained for SCA and composites except in the case of SPCL+20%HA which was still comparable with PS.

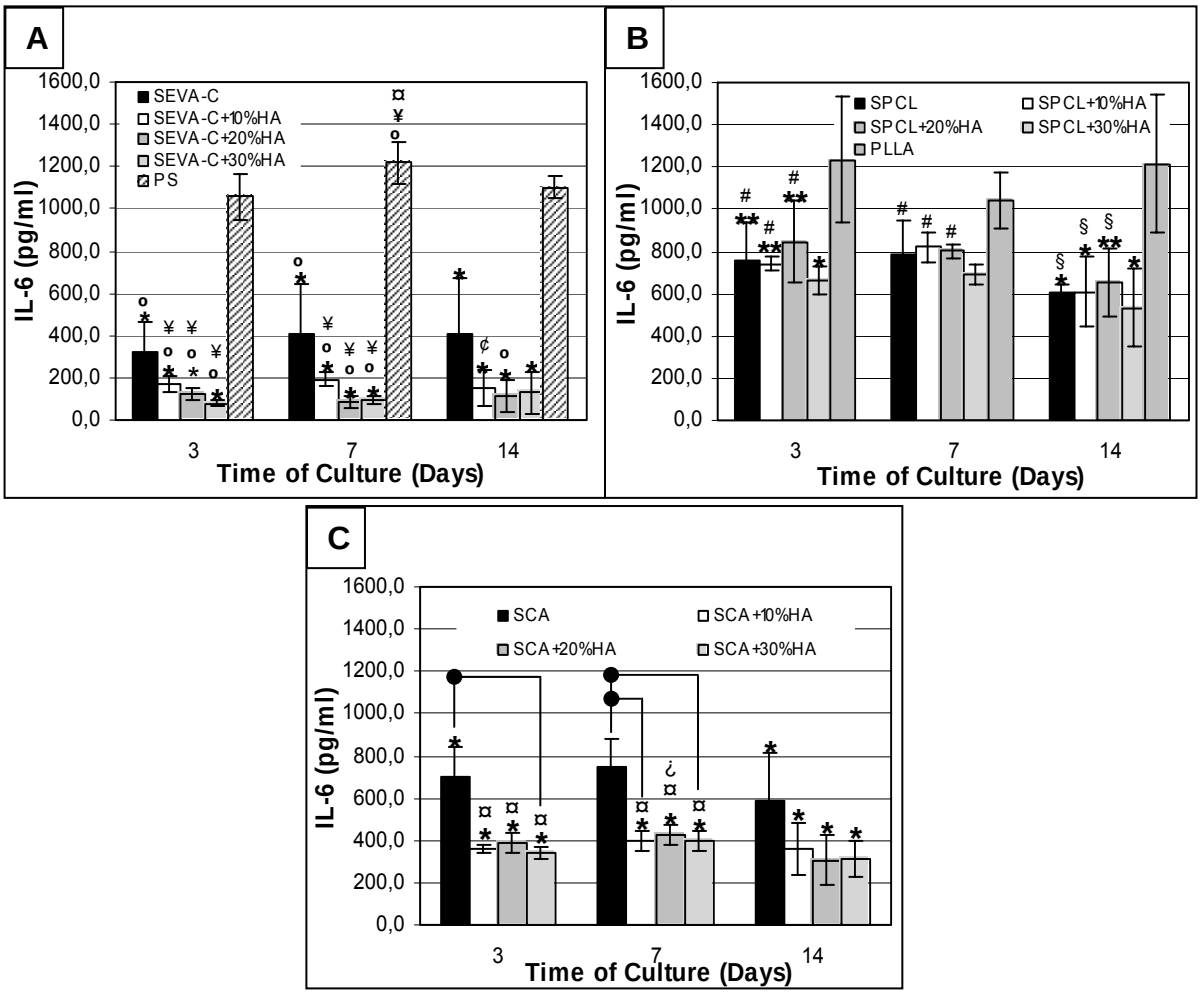

Figure 7.2. IL-6 release from monocytes/macrophages and lymphocytes when in culture with (a) SEVA-C and respective composites with HA and PS, (b) SPCL and respective composites with HA and PLLA, (c) SCA and respective composites with HA, for 3, 7 and 14 days. Data represents mean ± standard deviation, n≥3. *Indicates significant difference when comparing with PLLA and PS. **Indicates significant difference when compared with PLLA. ^{o}Indicates significant difference when compared with SCA. $^{\#}$Indicates significant difference when compared with SEVA-C and respective composites. §Indicates significant difference when compared with SEVA composites with 20% and 30% of HA. $^{¿}$Indicates significant difference when compared with SEVA-C+20%HA. $^{¤}$Indicates significant difference when compared with SPCL and respective composites with 10% and 20%HA. $^{¢}$Indicates significant difference when compared with SPCL+20%HA. $^{¥}$Indicates significant difference when compared with SPCL+30%HA. ●—Indicates significant difference between the connected bars.

SEVA-C and composites seemed to stimulate less leukocyte activation (Fig. 7.2a). The amounts of IL-6 detected in the presence of those materials was lower than those obtained with any of the other materials without any differences being measured between polymer and composites although a decrease was observed with increasing HA percentages. After 3 days

of culture these were found to be statistically different from SPCL and it's composites with 10%HA, 20%HA and SCA, as well as the results between SPCL+30%HA and SEVA-C composites, only changing after 7 days when comparing SEVA-C+30%HA and SCA+20%HA. After 14 days of culture, the results for SEVA and composites and SCA became closer and only SEVA-C+20%HA was found to induce significant IL-6 production when compared with SCA. Also when compared with SPCL and it's composites with 10%HA and 20%HA, only SEVA-C reinforced with 20%HA and 30%HA were found to be different. Furthermore the SEVA-C+10%HA result was also different from SPCL+20%HA.

Following SEVA-C and composites, SCA and respective composites were the materials that induced the production of IL-6 (Fig. 7.2c). Again, the same trend, in terms of the amount of released cytokine and percentage of HA was observed. However, some statistical differences were found after 3 days of culture between SCA and the composite with 30%HA and after 7 days of culture between SCA and the composites with 10%HA and 30%HA. Furthermore, at 3 and 7 days of culture it was observed that the results in the presence of SCA composites were different from the results in the presence of SPCL composites.

Table 7.2: Statistical summary for IL-6 expression for different pair of polymers (Statistically significant p values)

Time Culture	Material	SEVA-C	SEVA-C + 10%HA	SEVA-C + 20%HA	SEVA-C + 30%HA	SPCL	SPCL + 10%HA	SPCL + 20%HA	SCA	PS	PLLA
3 Days	SEVA-C									0	0
	SEVA-C+10%HA									0	0
	SEVA-C+20%HA									0	0
	SEVA-C+30%HA									0	0
	SPCL	0.004	0	0	0						0.006
	SPCL+10%HA	0.007	0	0	0						0.004
	SPCL+20%HA	0	0	0	0						0.045
	SPCL+30%HA		0.01	0	0					0.013	0.001
	SCA	0.022	0							0.032	0.001
	SCA+10%HA					0.012	0.018	0.001		0	0
	SCA+20%HA					0.027	0.041	0.003		0	0
	SCA+30%HA					0.007	0.011	0.001	0.037	0	0
7 Days	SEVA-C									0	0
	SEVA-C+10%HA									0	0
	SEVA-C+20%HA									0	0
	SEVA-C+30%HA									0	0
	SPCL	0.017	0	0	0					0.005	
	SPCL+10%HA	0.007	0	0	0					0.012	
	SPCL+20%HA	0.011	0	0	0					0.007	
	SPCL+30%HA		0.001	0	0					0	
	SCA	0.046	0	0	0					0.002	
	SCA+10%HA					0.015	0.006	0.010	0.042	0	0
	SCA+20%HA			0.050		0.031	0.012	0.021		0	0
	SCA+30%HA					0.014	0.006	0.009	0.040	0	0
14 Days	SEVA-C									0	0
	SEVA-C+10%HA									0	0
	SEVA-C+20%HA									0	0
	SEVA-C+30%HA									0	0
	SPCL			0.032	0.042					0.025	0.010
	SPCL+10%HA			0.029	0.038					0.028	0.011
	SPCL+20%HA		0.025	0.012	0.016						0.024
	SPCL+30%HA									0.007	0.003
	SCA			0.041						0.020	0.008
	SCA+10%HA									0	0
	SCA+20%HA									0	0
	SCA+30%HA									0	0

In the presence of SPCL and composites, in contrast with the other starch-based materials, it was possible to detect and quantify almost 3 times more IL-6. The amount of HA did not seem to have any influence (Fig. 7.2b).

7.3.3 IL-1β

IL-1β production was material dependent and in some cases with the time of culture. SEVA-C and composites, as well as the SCA composites did not show any differences with the time of culture. However, SCA and PS showed significant decreases in IL-1β production with increasing time of culture. The amount of cytokine released in the presence of SPCL with 10%HA and 20%HA also showed that tendency, although in the case of the composite with 10%HA, the difference was only statistically significant after 14 days. IL-1β was detected in very low amounts after contact with the starch-based materials when compared to the control materials (Fig. 7.3). In fact after 3 and 7 days of culture, all the materials, except for SCA and SPCL ($p<0.05$ only when compared with PS) showed a significantly different result from PS and PLLA. After 14 days of culture, almost no IL-1β was detected in the supernatants.

Table 7.3: Statistical summary for IL-1β expression for different pair of polymers (Statistically significant p values)

Time Culture	Material	SPCL + 20%HA	SCA	PS	PLLA
3 Days	SEVA-C		0	0	0
	SEVA-C+10%HA	0.017	0	0	0
	SEVA-C+20%HA	0.017	0	0	0
	SEVA-C+30%HA	0.049	0	0	0
	SPCL		0	0	0
	SPCL+10%HA		0	0	0
	SPCL+20%HA		0	0	0
	SPCL+30%HA		0	0	0
	SCA			0	0
	SCA+10%HA		0	0	0
	SCA+20%HA		0	0	0
	SCA+30%HA	0.017	0	0	0
7 Days	SEVA-C		0.025	0	0.01
	SEVA-C+10%HA		0.010	0	0
	SEVA-C+20%HA			0	0.010
	SEVA-C+30%HA		0.010	0	0
	SPCL			0.022	
	SPCL+10%HA			0	0.002
	SPCL+20%HA			0.001	0.030
	SPCL+30%HA			0	0.003
	SCA				
	SCA+10%HA		0.010	0	0
	SCA+20%HA		0.020	0	0.01
	SCA+30%HA		0.010	0	0

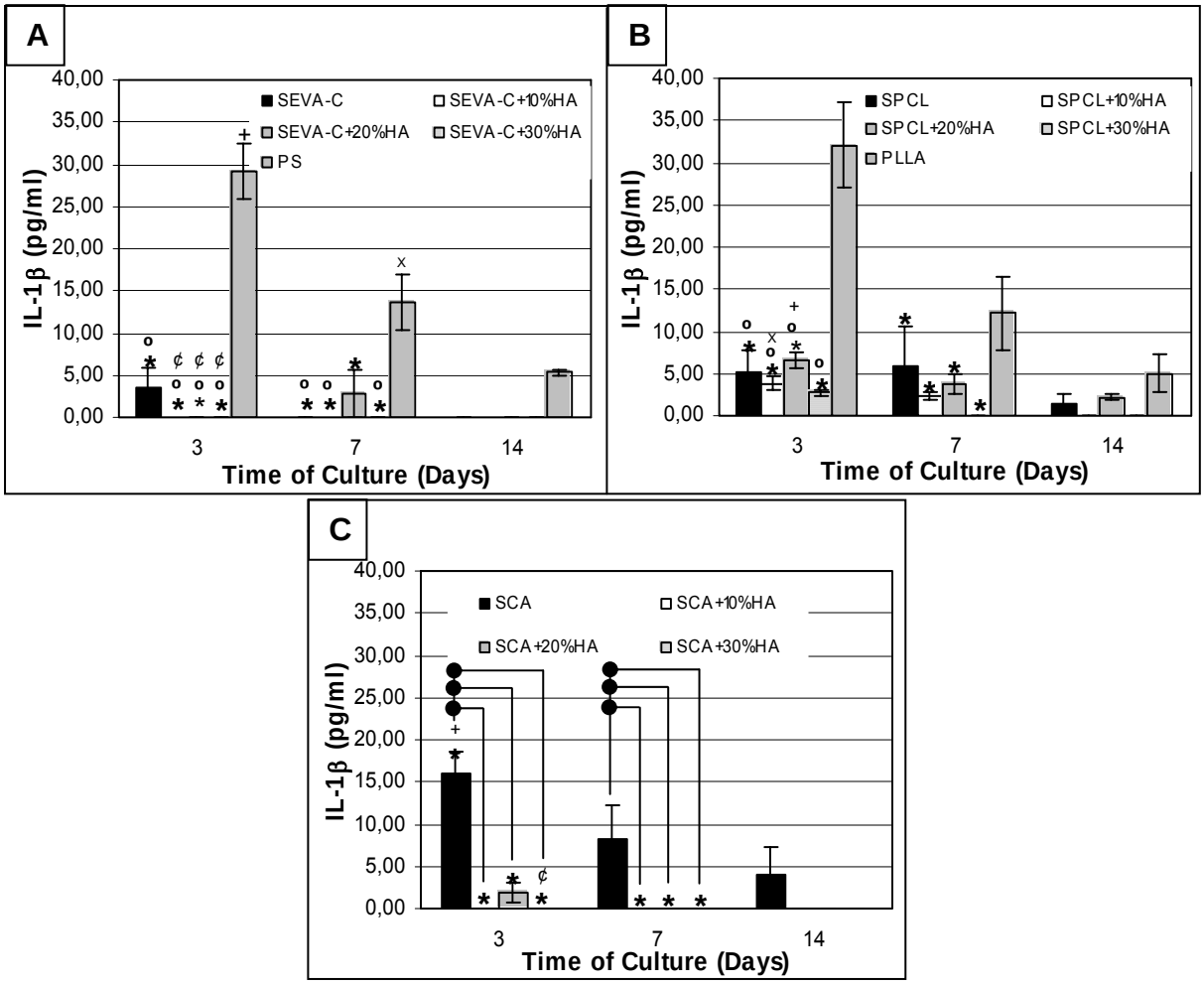

Figure 7.3. IL-1β release from monocytes/macrophages and lymphocytes when in culture with (a) SEVA-C and respective composites with HA and PS, (b) SPCL and respective composites with HA and PLLA, (c) SCA and respective composites with HA, for 3, 7 and 14 days. Data represents mean ± standard deviation, n≥3. *Indicates significant difference when compared with PLLA and PS. **Indicates significant difference when compared with PS. ^{o} Indicates significant difference when compared with SCA. $^{¢}$Indicates significant difference when compared with SPCL+20%HA. ●— Indicates significant difference between the connected bars.
$^{+}$ Indicates significant difference with 7 and 14 days of culture. ^{x} Indicates significant difference with 14 days of culture.

Comparing the amounts of IL-1β released in the presence of starch-based materials after 3 days of culture, SCA induced production in significantly different quantities (Fig. 7.3c). That difference was only maintained after 7 days of culture for SEVA-C and SEVA-C composites with 10%HA and 30%HA (Fig 7.3a) and for SCA composites (Fig. 7.3c).

Although only after incubation of cells with SPCL composites, HA was found not to reduce the amount of produced IL1-β, when compared with the non reinforced polymers. The amount was only different after 3 days and only between SPCL+20%HA and SEVA-C composites and SCA+30%HA.

7.3.4 TNF-α

The results obtained for TNF-α demonstrated significant variations according to the type of material, in some cases also being influenced by the percentage of ceramic filler and by the time of culture. Again, as for IL-6 the general tendency was a reduction in release with time of culture for most of the materials. For some of the materials there were detectable differences after 7 and 14 days of culture (SEVA-C+10%HA, PLLA), others only revealed variations in the amount of cytokine after 14 days in culture (SEVA-C, SEVA-C+20%HA, SPCL+10%HA and PS).

The level of TNF-α detected in the supernatants of the cultures was between 3 and 190 pg/ml (Fig. 7.4). The higher amounts of TNF-α were detected in the presence of PLLA and PS. After 3 days of culture in contact with all the materials and composites, the released TNF-α was found to be significantly different from PS and PLLA, except for SPCL+30%HA and SCA ($p<0.05$ only when compared with PLLA). After 7 days differences were observed for SEVA-C and composites, SPCL and SCA composites while the results for SPCL reinforced with 10%HA and 30%HA were similar to PS. After 14 days of culture only the SEVA-C composites were found to be different from the control materials. However, the amount of cytokine released during contact with SEVA-C, SPCL and SCA composites was still different from PLLA.

Analysing the amount of TNF-α in the presence of SEVA-C and composites a similar trend to IL-6 was observed, lower levels were detected when compared with all the other materials (Fig. 7.4a). Comparing the polymer and composites, it was found that the amount of cytokine released decreased with increasing percentages of HA. Furthermore, after 3 days of culture, the amount of TNF-α detected in the presence of SEVA-C was found to be significantly different for the result obtained with SEVA-C+30%HA.

Comparing the results obtained with SEVA-C and composites, with all the other materials, after 3 and 7 days statistically significant differences were found with SCA. After 14 days, only the difference between SEVA-C+30%HA and SCA remained. SEVA-C composites were found to be significantly different from SPCL composites after 3 and 7 days, except for SPCL+30%HA after 7 days ($p<0.05$ only when compared with SEVA-C with 20%HA and 30%HA). The amount of TNF-α released in contact with SEVA-C+30%HA was also found to be different from the SPCL composite with the same amount of HA at the 14 day time period.

When comparing all the starch-based materials, higher amounts of TNF-α were released in the presence of SPCL and composites (Fig. 7.4b). In this case, and contrary to SEVA and it's composites, increasing percentages of HA resulted in increasing amounts of cytokine release. In fact, and in addition to the statistical differences reported after 3 days in culture,

TNF-α released in the presence of SPCL was found to be different from the amount detected in the presence of SEVA-C with 20%HA and 30%HA.

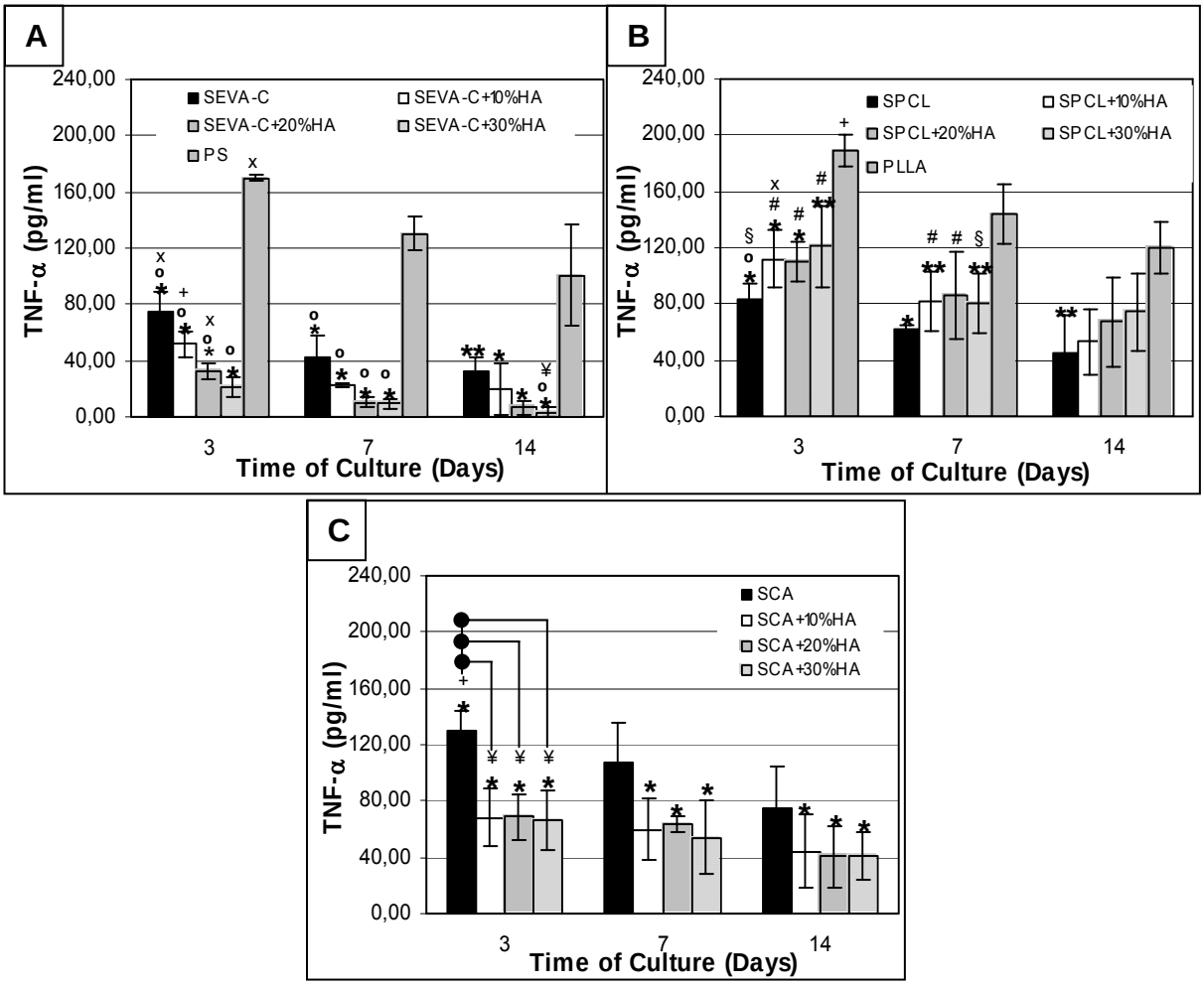

Figure 7.4. TNF-α release from monocytes/macrophages and lymphocytes when in culture with (a) SEVA-C and respective composites with HA and PS, (b) SPCL and respective composites with HA and PLLA, (c) SCA and respective composites with HA, for 3, 7 and 14 days. Data represents mean ± standard deviation, n≥3. *Indicates significant difference when compared with PLLA and PS. ^{o} Indicates significant difference when compared with SCA. $^{\#}$Indicates significant difference when compared with SEVA-C composites. §Indicates significant difference when compared with SEVA composites with 20% and 30% of HA. $^{¥}$Indicates significant difference when compared with SPCL+30%HA. ●——Indicates significant difference between the connected bars.
$^{+}$ Indicates significant difference with 7 and 14 days of culture. ^{x} Indicates significant difference with 14 days of culture.

Once again, and in agreement with IL-6 quantification, the level of TNF-α produced in contact with SCA composites was lower than that detected for SPCL composites and higher than the amount obtained for SEVA-C composites. However, in the case of the unreinforced polymer (SCA) the amounts detected were the highest when comparing with SEVA-C or SPCL.

Table 7.4: Statistical summary for TNF-α expression for different pair of polymers (Statistically significant p values)

Time Culture	Material	SEVA-C	SEVA-C + 10%HA	SEVA-C + 20%HA	SEVA-C + 30%HA	SPCL + 30%HA	SCA	PS	PLLA
3 Days	SEVA-C						0.011	0	0
	SEVA-C+10%HA						0	0	0
	SEVA-C+20%HA						0	0	0
	SEVA-C+30%HA	0.017					0	0	0
	SPCL			0.033	0.003		0.049	0	0
	SPCL+10%HA		0.005	0	0			0.034	0
	SPCL+20%HA		0.008	0	0			0.023	0
	SPCL+30%HA		0.01	0	0				0.001
	SCA								0.007
	SCA+10%HA					0.020	0.003	0	0
	SCA+20%HA					0.022	0.003	0	0
	SCA+30%HA					0.014	0.002	0	0
7 Days	SEVA-C						0.021	0	0
	SEVA-C+10%HA						0.001	0	0
	SEVA-C+20%HA						0	0	0
	SEVA-C+30%HA						0	0	0
	SPCL							0.010	0.001
	SPCL+10%HA		0.045	0.007	0.005				0.028
	SPCL+20%HA		0.022	0.003	0.002				
	SPCL+30%HA			0.009	0.002				0.023
	SCA								
	SCA+10%HA							0.007	0.001
	SCA+20%HA							0.014	0.002
	SCA+30%HA							0.003	0
14 Days	SEVA-C								0.004
	SEVA-C+10%HA							0.012	0.001
	SEVA-C+20%HA							0.002	0
	SEVA-C+30%HA						0.040	0.001	0
	SPCL								0.026
	SPCL+10%HA								
	SPCL+20%HA								
	SPCL+30%HA				0.043				
	SCA								
	SCA+10%HA								0.021
	SCA+20%HA								0.013
	SCA+30%HA								0.014

Contrary to any of the other starch-based blends, the presence of HA in the starch blend with cellulose acetate induced a decrease in the amount of TNF-α production although the percentage of ceramic had no influence. After 3 days of culture, the results obtained for SCA were found to be different from those obtained for composites. In addition, the results for composites were also different from the result obtained for SPCL+30%HA.

7.4. DISCUSSION

Several studies[31,32] have demonstrated that leukocytes attach and adhere to biomaterial surfaces. Upon adherence, those cells undergo morphological and physiological changes such as membrane perturbation and frustrated phagocytosis that can lead to cell activation and the release of chemical mediators such as cytokines.[1,32-34] These cytokines can influence the initiation, duration and resolution of the host response to injury, which is determined by the prolonged presence of the biomaterial.[35]

It has been reported that there are several factors that modulate the cellular activation process and mediate production of cytokines. Surface physical and chemical properties[36-38] and adhesion specific signals[31,36,37] are believed to have an important role, however, some authors[39] defend that unknown factors also might be involved in monocyte differentiation and activation.

IL-1 β, TNF-α and IL-6 were considered as markers for polymer-induced macrophage activation and it was shown that starch-based polymers and composites significantly reduce the release of these cytokines compared to PS and PLLA.

It was reported by Chomyszyn-Gajewska et al[40] that in the absence of serum, PLLA did not stimulate the release of IL-6, IL-12 and TNF-α. However, following an initial incubation with serum, the release of IL-6 and IL-12 increases dramatically, which was related to the fact that the studied materials have no surface ligands recognisable by macrophages to trigger the synthesis of certain cytokines.

Activation of monocytes may occur following adhesion to a surface, or alternatively, through cell-cell or cell-mediator interaction without adhesion to the surface. Studies with tissue culture polystyrene found the greatest release of IL-1β, IL-6 and TNF-α by monocytes/macrophages cultured in contact with that polymer in comparison with other polymers. Ung et al[41] assumed that the surface treatment performed in tissue culture plates to achieve maximum adhesion leads to increasing activation of monocytes/macrophages.

In the present work, cytokines were determined in the cell supernatant, therefore the detected levels reflected the combination of adherent and nonadherent cells. We have previously demonstrated (paper in press)[30] that the number of cells adhered to the surface of PS was lower than in the case of certain starch-based polymers and composites. Therefore, we may speculate that non-adherent cells had a major contribution in the production of pro-inflammatory cytokines in the presence of PS.

Khouw et al[42] found that applying monoclonal antibodies against IFN-γ inhibited the foreign body reaction to hexamethylenediisocyanate which indicated the important role played by that cytokine in the inflammatory process as a well as the possibility to modulate its activity. Some authors[43] defend that macrophages, besides NK cells and T lymphocytes, are also able to produce IFN-γ however, T cell inhibition results in a foreign body reaction delay which could suggest an active role of T-lymphocytes in the host response.

Although the polymers in this study were cultured with a mixed population of monocytes/macrophages and lymphocytes, T-cells did not demonstrate significant activation.

TNF-α has a crucial role in the inflammatory response, resulting in a great number of cases in osteolysis around the implant.[1,2,5,6] Several studies found that this cytokine can be

produced at much higher levels than IL-6 and IL-1β by macrophages in the presence of polymer or metal particles.[44-46]

The regulation of TNF-α production is complex and can be inhibited by IL-4, IL-10 or IL-13 and greatly enhanced by IFN-γ. Levings et al[47] proved that IL-4-mediated inhibition of TNF-α release occurs by a transcription factor STAT6-dependent mechanism. However, in the presence of IFN-γ, another and physiologically more important STAT6-independent mechanism is active. In this study, no IFN-γ was detected and the amount of produced IL-4 was similar in the presence of all materials. Therefore it might be considered that IL-4 has no additional effect in the TNF-α released in the presence of the studied materials.

TNF-α and IL-6 were released in higher amounts and with an almost similar distribution, which may reflect monocyte differentiation, as IL-6 has been implicated in monocyte differentiation[19] and TNF-α has been described as an autocrine regulator of macrophage differentiation[48].

IL-1β is produced in response to many stimuli which include bacterial LPS, numerous microbial products, cytokines (TNF-α, IFN-γ, GM-CSF and IL-2), T-cell/antigen presenting cell interactions and immune complexes and was found to stimulate the production of IL-6 in peripheral blood monocytes.[49]

The IL-1β production detected was zero or minimal in the presence of some materials, suggesting resting or non-stimulated monocytes and macrophages. It seems that no cross effect was happening between TNF-α release and IL-1β production or that the amount of released TNF-α was not enough to stimulate IL-1β production. Furthermore, the amount of detected IL-6 was significantly higher than the amount of IL-1β.

It has been reported that the hydrophilic/hydrophobic character of a polymer surface, characterised by the contact angle, may influence cytokine production from monocytes[36] however, other factors may also be important, since other studies have reported that similar compositions and contact angles result in different IL-1 production[39]. Yun et al[37] reported that specific surfaces like hydrophilic and neutral surfaces may be the least likely to adhere and activate monocytes.

In fact starch-based polymers have different surfaces wettabilities. The more hydrophilic surfaces (SCA) induced higher TNF-α and IL-1β production while SPCL, the polymer with the most hydrophobic surface together with SCA showed the highest secretion of IL-6. It could be speculated that in the case of IL-6, hydrophilicity is not the most important factor for monocyte/macrophage activation, while the TNF-α and IL-1β production seems to be dependent on and more evident in more hydrophilic surfaces.

Protein adsorption can directly influence the activation of monocytes and macrophages on a surface in a time dependent manner. DeFife et al[31] found that fibrinogen and IgG

preadsorption resulted in different IL-1β, IL-6 and TNF-α concentrations in the supernatants depending on the polymers and the time of culture. Previous studies with starch-based polymers and human serum demonstrated that SPCL was the polymer that presented the highest levels of protein adsorption.[50] If protein adsorption implies cell adhesion and consequently cell activation, the results obtained, in terms of IL-1β and TNF-α production, did not support this theory.

Concerning the HA reinforcement, although some authors report improved performance of those implants in terms of femoral remodelling and bone density[51,52], others report that ceramic coatings may produce particulate wear debris and osteolysis[53,54]. Ninomiya et al[5] reported that HA enhanced the production of IL-1β, IL-6 and TNF-α by human fibroblasts *in vitro*. In the present study HA resulted in a significant reduction of those inflammatory cytokines, especially in the case of SCA and for shorter culture periods. SEVA-C composites also showed a lower effect in the secretion of IL-6 and TNF-α while in the case of SPCL composites the only evident effect was the secretion of TNF-α, in this case, HA induced higher production of TNF-α.

7.5 CONCLUSIONS

The isolation and culture of a mixed population of mononuclear cells enabled the evaluation of the inflammatory potential

Starch-based polymers and composites did not elicit a very strong reaction from immune system cells in vitro, demonstrated by a lower production of cytokines when compared to PLLA biodegradable material.

HA reinforcement resulted in lower cell activation, potentially enabling better mechanical properties to be combined with reduced levels of cytokine production.

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CHAPTER 8

AN *IN VIVO* STUDY OF THE HOST RESPONSE TO STARCH-BASED POLYMERS AND COMPOSITES SUBCUTANEOUSLY IMPLANTED IN RATS

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ABSTRACT

Implant failure is one of the major concerns in the biomaterials field. Several factors have been related to the fail but in general these biomaterials do not exhibit comparable physical, chemical or biological properties to natural tissues and ultimately, these devices can lead to chronic inflammation and foreign body reactions.

Starch-based biodegradable materials and composites have shown promising properties for a wide range of biomedical applications as well as a reduced capacity to elicit a strong reaction from immune system cells *in vitro*. In this work, blends of corn starch with ethylene-vinyl alcohol (SEVA-C), with cellulose acetate (SCA) and polycaprolactone (SPCL), as well as hydroxyapatite (HA) reinforced starch-based composites, were subcutaneously implanted in rats.

The aim of the work was to assess the host response evoked for starch-based biomaterials, identifying the presence of important cell types. The tissues surrounding the implant were harvested together with the material and were analysed using immunohistochemistry. Markers for resident and recruited macrophages as well as for T lymphocytes were used in order to identify the types of cells and their subpopulations present in the implant area. Furthermore markers for activated macrophages and for antigen presenting cells expressing Major Histocompatibility Complex (MHC) class II molecules were used to try to understand the intensity of the tissue reaction.

At implant retrieval there were no macroscopic signs of a considerable inflammatory reaction in any of the animals; no cellular exudate was formed around the implants. A thin fibrous capsule, invariably containing inflammatory cells ranging from diffuse to concentrated density surrounded all implants. The histological analysis of the interface tissue after

immunohistochemistry using ED1, ED2, CD54, MHCII and α/β antibodies showed positively stained cells for all antibodies, except for α/β for all the implantation periods, where it was different for the various polymers and for the period of implantation. The presence of blood vessels was also observed in the majority of the cases.

SPCL and respective composites were the materials that stimulated the stronger tissue responses but generally biodegradable starch-based materials did not induce a severe reaction for the studied implantation times which contrasts to other types of degradable polymeric biomaterials.

8.1. INTRODUCTION

The challenge in the development of new devices for orthopaedics is to ensure long-term stability, anchorage and function. Loosening of joint prosthesis resulting in failure is a major concern in the biomaterials field for orthopaedic applications[1,2] with revision surgery occurring at both early and late implantation periods depending on the cause of failure. Unpredictable adverse reactions to some commonly used traditional implants have been reported during the years[3-7]. Key factors are believed to be the generation of wear particles and the biological response to them in periprosthetic tissues[2,4] as well as the degradation products of biodegradable materials which result in osteolytic reactions[5,6,8].

The presence of activated macrophages[9-13], foreign body giant cells[9,11,13] and the formation of fibrous capsule[14,15] are tissue-specific responses that have been the focus of investigation in the evaluation of biomedical implants. Additionally, the evaluation of angiogenesis[12,13,16,17] in the implant area has been realised as an important factor which may significantly influence the polymer-tissue interface. The presence of giant cells is frequently observed[9,11,13] and if the duration and severity of the reaction may or may not compromise the role of the device.. Phagocytic cells, normally involved in inflammation are thought to be responsible for removing the final products of degradation. In fact, both clinical applications[4-6] and animal studies[18-21] have suggested that degradation products directly and indirectly affect tissue remodelling respectively by interaction with the cells responsible for the formation of *de novo* tissue and through the induction of inflammatory cytokines released by activated macrophages, particle size has therefore been suggested as an important factor in the different tissue reactions. Macrophages tend to engulf smaller particles[22] and form multinuclear giant cells to surround larger objects[23] although the differences in duration of the response may also be related to the material properties[23]. The duration of the inflammatory reaction has also been correlated with the angiogenesis around an implant [12,13,16,17]. A co-dependence has been proposed between inflammation and angiogenesis[24,25]. Activated macrophages are capable of releasing numerous angiogenic growth factors[24,26]which may be

responsible for the angiogenesis around an implant. The up-regulation of adhesion molecules is known to have a significant role in the process of transvascular migration of the inflammatory infiltrate[27,28].

Lymphocytes have also been observed at the interface of some implants[18,29-31]. These cells are able to secrete various mediators which, in turn, have functions in immunological and inflammatory responses. Although in the majority of the cases they are identified in low numbers, lymphocytes may secrete IL(interleukin)-4[32] and interferon (IFN)-γ[31] which can induce macrophage fusion and activation. Serious complications have been demonstrated when lymphocytes were the main type of cell found in a retrieved cell suspension with a low number of mononuclear phagocytes[29], which suggested a lymphocyte-mediated specific immunological reaction against the implant. Studies with T-cell deficient rats[30] have shown that T cells play a major role in the formation of giant cells and in the phagocytic activity of macrophages and giant cells during the tissue response to biomaterials. Presenting the possibility the tissue reaction to biomaterials might be modulated by controlling T-cell activation in the case of unwanted or secondary reactions, or in the case of too-fast degradation of biomaterials.

Starch-based biodegradable biomaterials, proposed for several biomedical applications[33-36], have also been shown to be degraded by α-amylase[37-39] and phagocytosed by macrophages[38,39]. This process has demonstrated an excellent low inflammatory tissue reaction when implanted both in rats and mice[39,40]. In works by other groups[41,42] starch-based materials implanted in rabbits and goats performed well without adverse reactions. The host response to cross-linked high amylose starch (Contramid®) was found to be in accordance with the main phases of the inflammatory and foreign body responses to injuries caused by implanted devices[43-46]. After 4 months only a small residual scar was apparent macroscopically and was related to a less severe early reaction than a skin incision and closure with suture material sham[39].

In this work, starch-based biomaterials were subcutaneously implanted in rats for different time periods in order to evaluate their immunogenicity. The tissues surrounding the implant were harvested together with the material and were analysed using immunohistochemistry. Markers for resident and recruited macrophages as well as for T lymphocytes were used in order to identify the types of cells and their subpopulations present in the implant area. Furthermore markers for activated macrophages and for antigen presenting cells expressing Major Histocompatibility Complex (MHC) class II molecules were used in order to try to understand the intensity of the tissue reaction.

8.2. MATERIALS AND METHODS

8.2.1 Materials

The materials studied were: i) a 50/50 (wt %) blend of corn starch and ethylene vinyl alcohol (SEVA-C, Novamont, Italy), ii) a 50/50 (wt %) blend of corn starch and cellulose acetate (SCA, Novamont, Italy), iii) SCA reinforced with 10%, 20% and 30% (wt) of hydroxyapatite, iv) a 30/70 (wt %) blend of corn starch and polycaprolactone (SPCL, Novamont, Italy) and v) SPCL reinforced with 10%, 20% and 30% (wt) of hydroxyapatite. In the composites the average size of 90% of the HA particles was found to be below 6.5 μm (laser granulometry analysis).

All the materials were processed by injection moulding under optimized processing conditions. Samples were cut into rectangular-shaped blocks 13mm x 10mm x 7mm and a hole, with 5mm diameter and 10 mm length was drilled (Fig. 8.1 A and B). Before implantation the edges of the samples were trimmed and samples were rolled for 1 week in glass flasks to round machined edges and reduce the magnitude of edge effects.

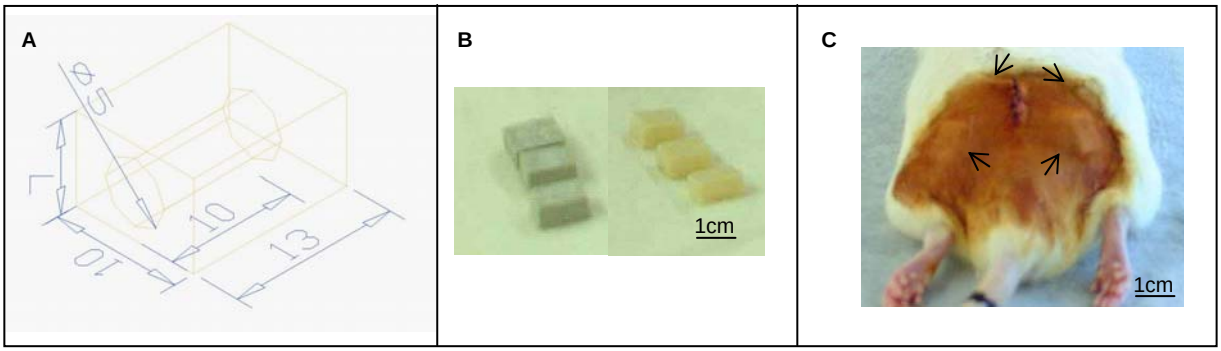

Figure 8.1. (A) Schematic representation of the implanted materials (scale in cm); (B) Two of the materials implanted; (C) Implant positions in the back of the rat (arrows).

8.2.2 Animals and Subcutaneous Implantation

The experiments were performed in Wistar rats, anaesthetized using Immobilon. Four different materials were implanted subcutaneously in the back, two either side of the spine, for 7, 14 and 21 days, with three repeats for each material per time period (Fig. 8.1C). Different positions and combinations of materials for each animal were performed to control site specific responses and the potential effect of degradation products. At the end of the implantation period, rats were sacrificed by CO_2 and the tissue surrounding the implant was carefully dissected and snap frozen using isopentane in cardice and stored at -80°C until sectioned.

8.2.3 Immunohistochemistry

Serial sections (7μm) were obtained at -20°C using a 5040 Microtome (Bright, England), sections were mounted in 3-aminopropyl-triethoxysilane (APES) coated slides, fixed with acetone for 5 minutes, air dried and kept at 4°C until staining.

Tissue sections were washed with phosphate buffer saline (PBS) solution and stained using an avidin-biotin alkaline phosphatase technique[47]. Materials were exposed to rabbit serum for 30 minutes to reduce nonspecific reactivity, followed by primary antibodies for 45 minutes at room temperature. After that time materials were rinsed with PBS for 5 minutes and incubated with biotinylated rabbit anti-mouse IgG antibody (Dako A/S, Denmark) for 1 hour at room temperature. The Avidin and Biotinylated horseradish peroxidase complex (Vector Laboratories Ltd., UK) was added to all materials for 1 hour and the substrate reaction was developed using the Alkaline Phosphatase Substrate Kit (Vector Laboratories Ltd., UK). Each incubation, except the rabbit serum, was followed by one wash with PBS buffer for 5 minutes. Materials were washed and counterstained with haematoxylin and mounted in permanent aqueous mounting medium (Serotec Ltd, UK). Each material had one sample stained as a control replacing the primary antibody with PBS buffer.

8.2.4 Antibodies

Individual leukocyte cell surface molecules were identified using the following panel of mouse anti-rat monoclonal antibodies: α/β (Serotec, UK) to targeting the α/β T cell antigen receptor found in 97%of peripheral T lymphocytes, ED1 (Serotec, UK) labelling monocytes and immature macrophages, ED2 (Serotec, UK) specific for resident/mature macrophages, CD54 y(Pharmingen, USA), which reacts with intracellular adhesion molecule-1 (ICAM-1) expressed in activated macrophages and HLA-DR antibody (Serotec, UK) which recognizes MHC II antigen present in activated macrophages and B lymphocytes.

8.3. RESULTS

At implant retrieval there were no macroscopic signs of a considerable inflammatory reaction in any of the animals; no cellular exudate was formed around the implants.

A thin fibrous capsule, invariably containing inflammatory cells ranging from diffuse to concentrated density surrounded all implants. The histological analysis of the interface tissue after immunohistochemistry using ED1, ED2, CD54, MHCII and α/β antibodies showed positively stained cells for all antibodies, except for α/β for all the implantation periods, where it was different for the various polymers and for the period of implantation. The presence of blood vessels was also observed in the majority of the cases.

8.3.1 SEVA-C

SEVA-C composites were not used in this study in order to keep a reasonable number of animals and consequently conditions and due to the in vitro results, which suggested being similar to the unreinforced polymer SEVA-C. The starch-ethylene vinyl alcohol blend (SEVA-C) showed, within the studied implantation period, a mild inflammatory reaction (Fig. 8.2).

A moderate cellular infiltration composed of macrophages was observed at the tissue-material interface for all periods of implantation. Recruited macrophages identified using the ED1 antibody were found in moderate amounts and mainly located in the tissue close to the interface with the material (Fig. 8.2A). After 21 days of implantation however, the staining increased indicating denser cellularity (Fig. 8.2B). Contrarily to recruited macrophages, tissue macrophages (ED2 positively stained) were in considerable numbers but dispersed in the surrounding outer layers of tissue (Fig. 8.2C).

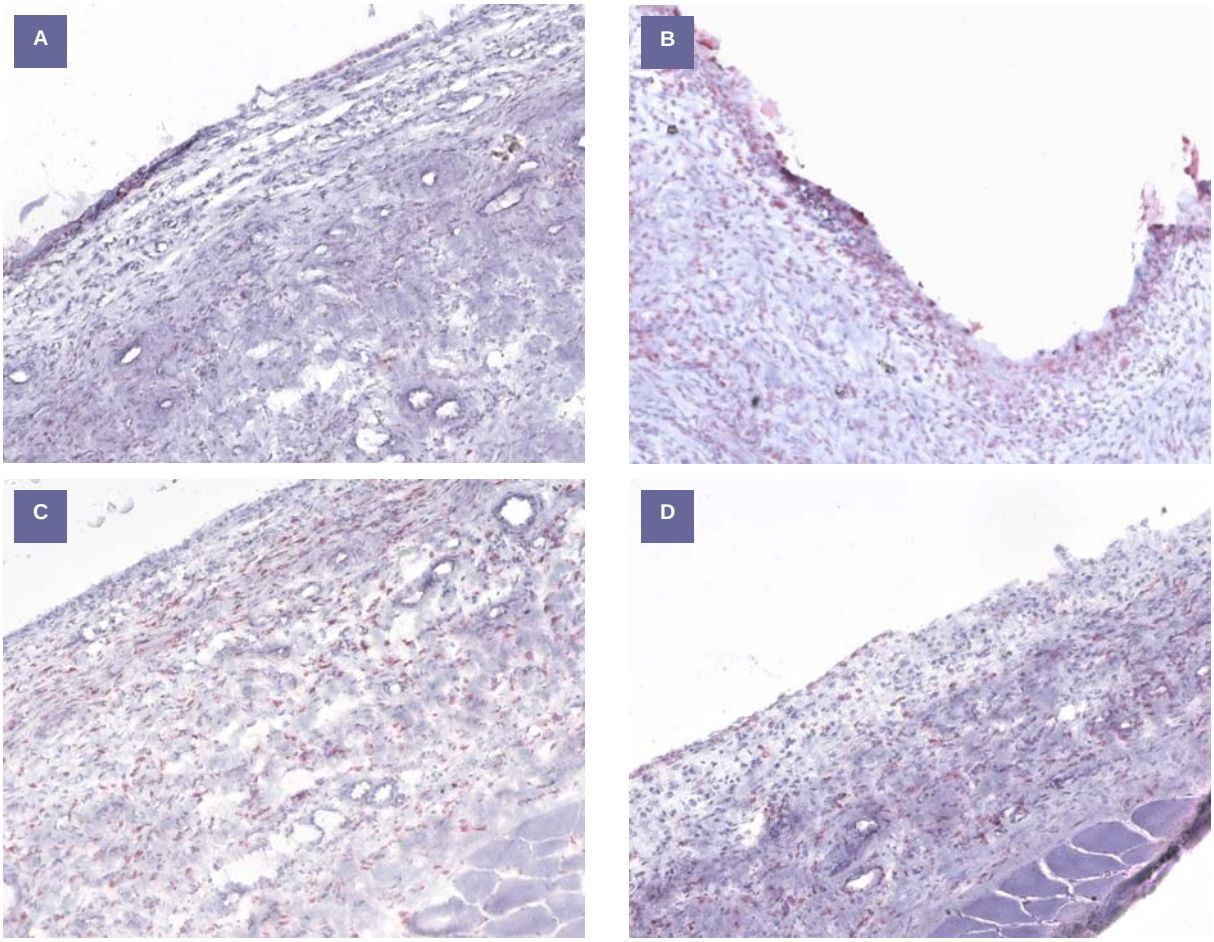

Figure 8.2. Inflammatory response to SEVA-C. Light micrographs of sections immunocytochemically stained for ED1 (A, B); ED2 (C) and MHCII (D). Explants shown here were taken after 14 (A, C, D) and 21 (B) days. Magnification (x10)

Antigen presenting cells (APC) expressing MHC class II molecules were distributed throughout the tissue surrounding the implant and also at the tissue-material interface

suggesting that these cells can belong to either macrophage sub- population (Fig. 8.2D). As for ED1 macrophages at 21 days of implantation the MHC class II staining pattern seems to be more intense.

A number of activated macrophages were also identified through the expression of ICAM-1. Contrarily to what might be expected, due to an increased intensity of the MHC II staining, the amount of cells expressing ICAM-1 did not seem to change over time. CD54 positive cells were defining the tissue-material interface, like the ED1 macrophages, but were also dispersed in the surrounding tissues. In addition, cells positive for CD54 were found to be adjacent to blood vessels at 14 days of implantation which suggests the infiltration of inflammatory cells in that period of time.

Few T lymphocytes were found and these few were in the tissue surrounding SEVA-C at 21 days of implantation.

8.3.2 SCA and Composites

The blend of starch with cellulose acetate seemed to demonstrate the lowest level of inflammation for the studied implantation periods which correlates with in vitro results[48] showing a lower number of cells from a mixed population of monocytes/macrophages and lymphocytes and a reduced amount of activated macrophages on that material. No T lymphocytes were found at the interface or in the tissues adjacent to the implant. Low numbers of recruited and resident macrophages were observed, which was comparable for all the times of implantation. Like for SEVA-C, the staining pattern showed ED1 macrophages at the interface tissue-material (Fig. 8.3A) and ED2 macrophages in the outside layer of the tissue (Fig. 8.3B).

The cells expressing MHC class II antibody were, in the case of SCA (Fig. 8.3C), slightly different than those observed for SEVA-C (Fig. 8.2D). Comparing the staining pattern of ED1 and ED2 macrophages with MHC II positive cells, it can be suggested that some of the recruited and resident macrophages are expressing those molecules. This statement is particularly valid for 7 days of implantation (Fig. 8.3). For longer times the intensity of the staining at the interface (comparable to ED1 pattern) decreased.

Similar to the results obtained for SEVA-C, the amount of activated macrophages, expressing ICAM-1 was moderate. However, these cells were only present at tissue-material interface on day 7. In addition, from day 14, CD54 positive cells were defining blood vessels near the tissue-implant interface (Fig. 8.4), which could indicate the influx of inflammatory cells to the site of implant.

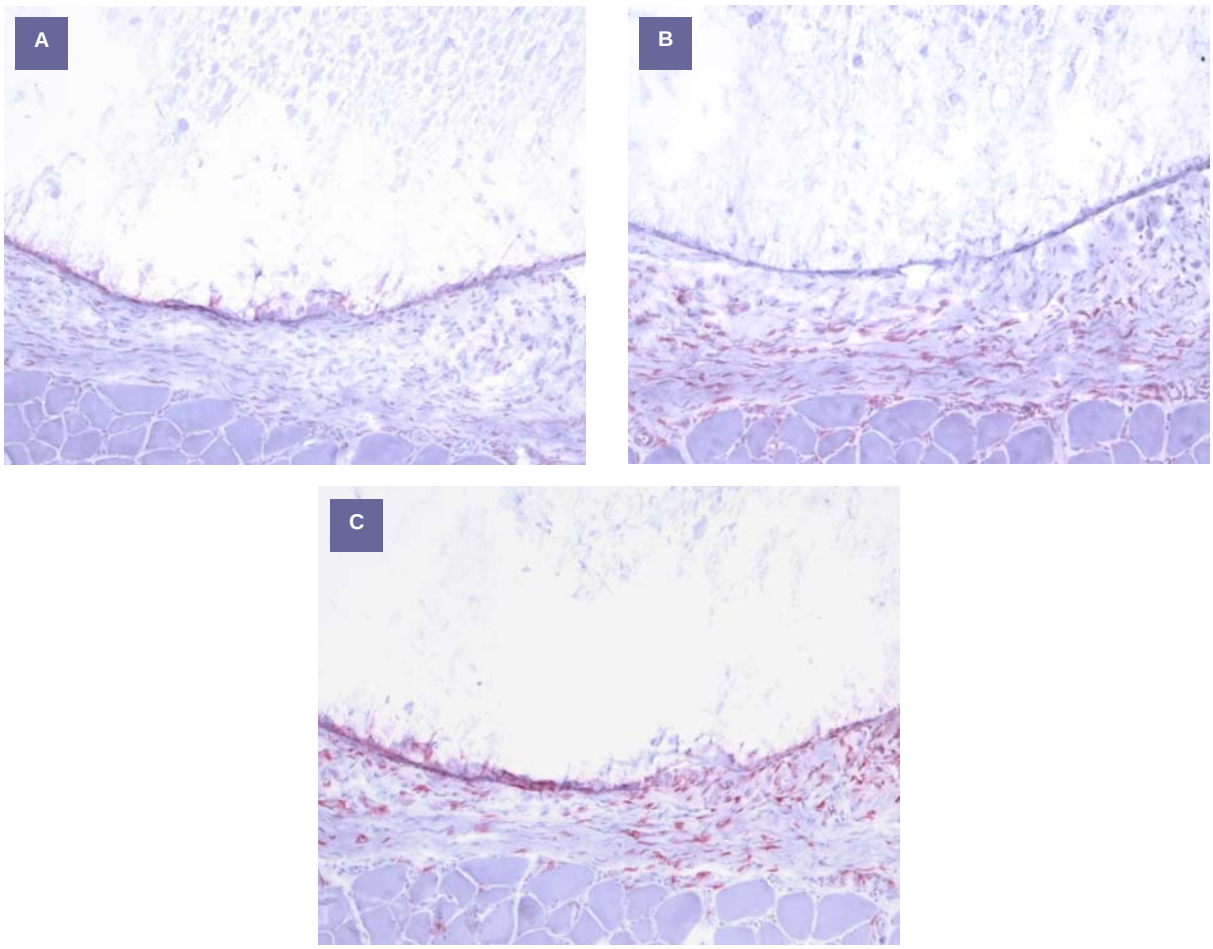

Figure 8.3. Inflammatory response to SCA. Light micrographs of sections immunocytochemically stained for ED1 (A); ED2 (B); MHCII (C). Explants shown here were taken after 7 days. Magnification (x10)

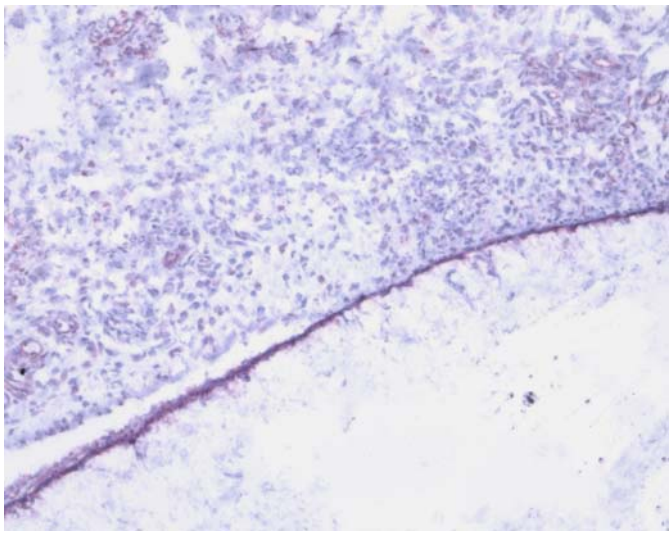

Figure 8.4. Expression of the adhesion molecule ICAM-1 in a section immunocytochemically stained for CD54. SCA was explanted at day 14. Positive macrophages and blood vessels are defined. Magnification (x10)

In terms of tissue reaction, the implantation of the SCA reinforced with HA, induced a greater effect. While in the presence of the SCA polymer no T lymphocytes were observed, for SCA composites the T cells which were recruited to the implantation site, although very few at day 7 remained there at 21 days (Fig. 8.5).

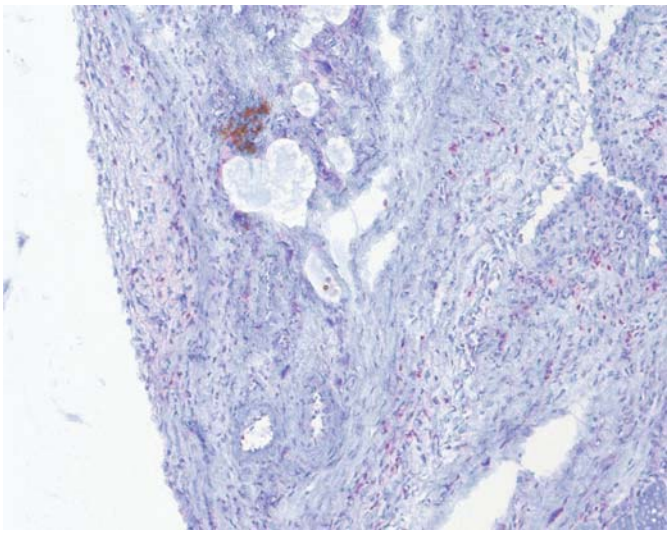

Figure 8.5. Expression of the α/β T cell antigen receptor in a section immunocytochemically stained for T cells. SCA+10%HA, day 21. Magnification (x10)

Surprisingly, higher percentages of HA seem to stimulate a greater tissue response. We might speculate that, since ED1 staining was more intense, the number of inflammatory cells attracted to the site of implantation of SCA composites was higher, particularly for 21 days of implantation (Fig. 8.6A). Furthermore, ICAM-1 expressing cells were found in the periphery of blood vessels from day 7 for SCA+20%HA (Fig. 8.6B) and SCA+30%HA (Fig. 8.6C) and only for 21 days of implantation in the case of SCA+10%HA (Fig. 8.6D).

The distribution pattern of recruited and resident macrophages did not show significant differences as compared with the other materials. ED1 positive cells were defining the interface, while ED2 positive cells were dispersed within the surrounding tissue. Cells expressing ICAM-1 however, were highly concentrated at the interface at the longer implantation periods (Fig. 8.6 B, C, D).

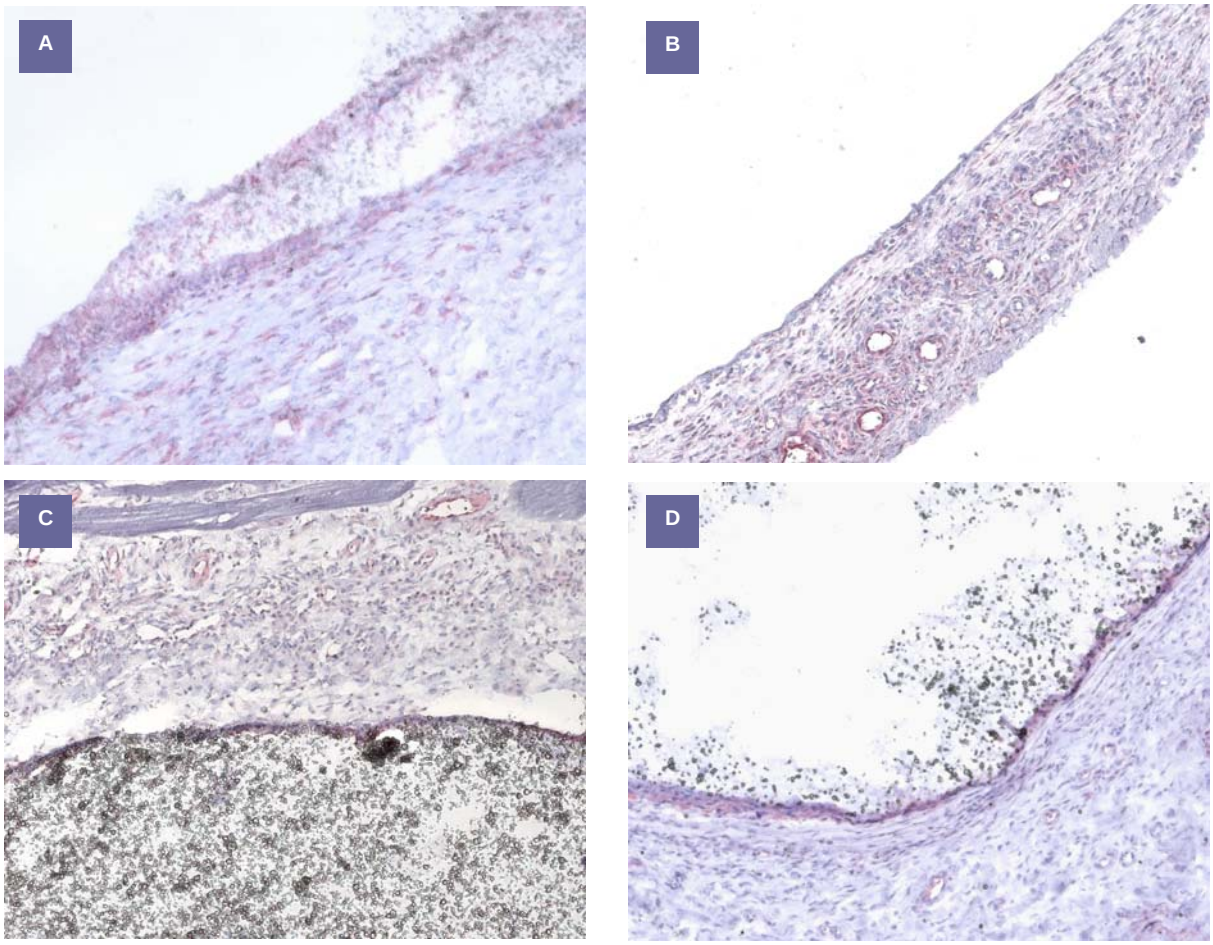

Figure 8.6. Inflammatory response to SCA composites;(A, D) SCA+10%HA; (B) SCA+20%HA; (C) SCA+30%HA. Light micrographs of sections immunocytochemically stained for ED1 (A); CD54 (B, C, D). Explants shown here were taken after (B, C) 7 and (A, D) 21 days. Magnification(x10)

The pattern of cells expressing MHC class II molecules was different in the presence of SCA composites compared to the unreinforced material, also showing variations for different percentages of HA. Thus, a greater number of cells extrapolated from the staining intensity, appear to express MHC class II after implantation of SCA with 20% and 30% of HA (Fig. 8.7A) comparatively to SCA+10%HA (Fig. 8.7B). Additionally, after 21 days of implantation of SCA+30%HA, the concentration of cells expressing MHC class II seems to increase being dispersed all over the tissue surrounding the implant Fig. 8.7C). Like for SCA, many of the cells expressing MHC II were probably macrophages especially due to the pattern similarity with the immunopositive macrophages ED1 at 21 days of implantation.

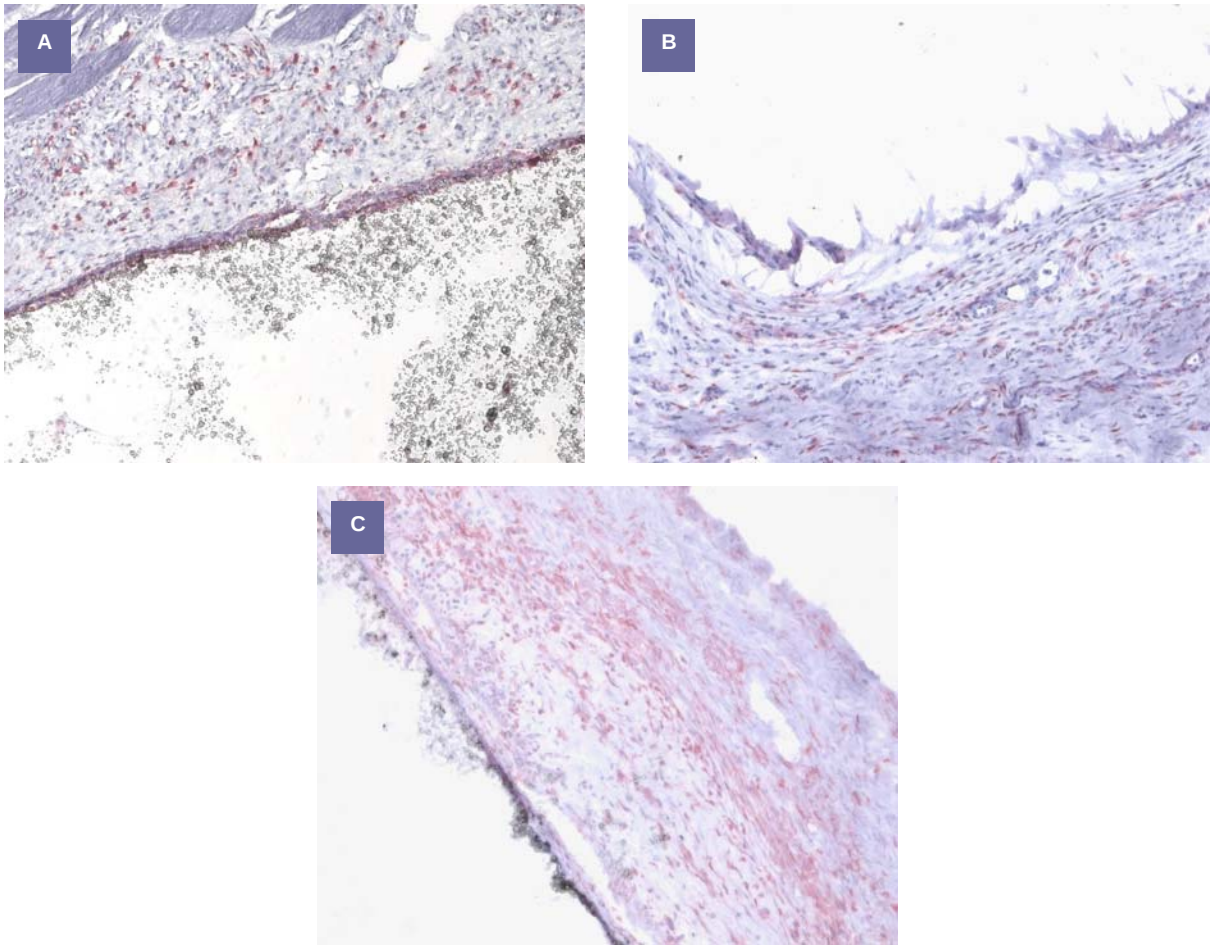

Figure 8.7. Expression of MHC class II molecule in sections immunocytochemically stained for antigen presenting cells. (A, C) SCA+30%HA; (B) SCA+10%HA. Explants shown here were taken after (A) 7 and (B, C) 21 days. Magnification (x10)

8.3.3 SPCL and Composites

The SPCL provoked a strong tissue reaction. ED1 macrophages were abundant at the SPCL interfaces from day 7 (Fig. 8.8A), persisting for the whole duration of the study. A high staining intensity of the cells expressing MHC class II molecules (Fig. 8.8B) was also observed. The highest amount of positive cell staining was observed for ED1 and MHC II antibodies in the sections obtained after 21 days of SPCL implantation.

Although not as abundant as ED1 and MHC II immunopositive cells, macrophages expressing ICAM-1 were also found in high intensity patterns. These were stronger for longer implantation times, demarcating blood vessels at 21 days of implantation (Fig. 8.8C).

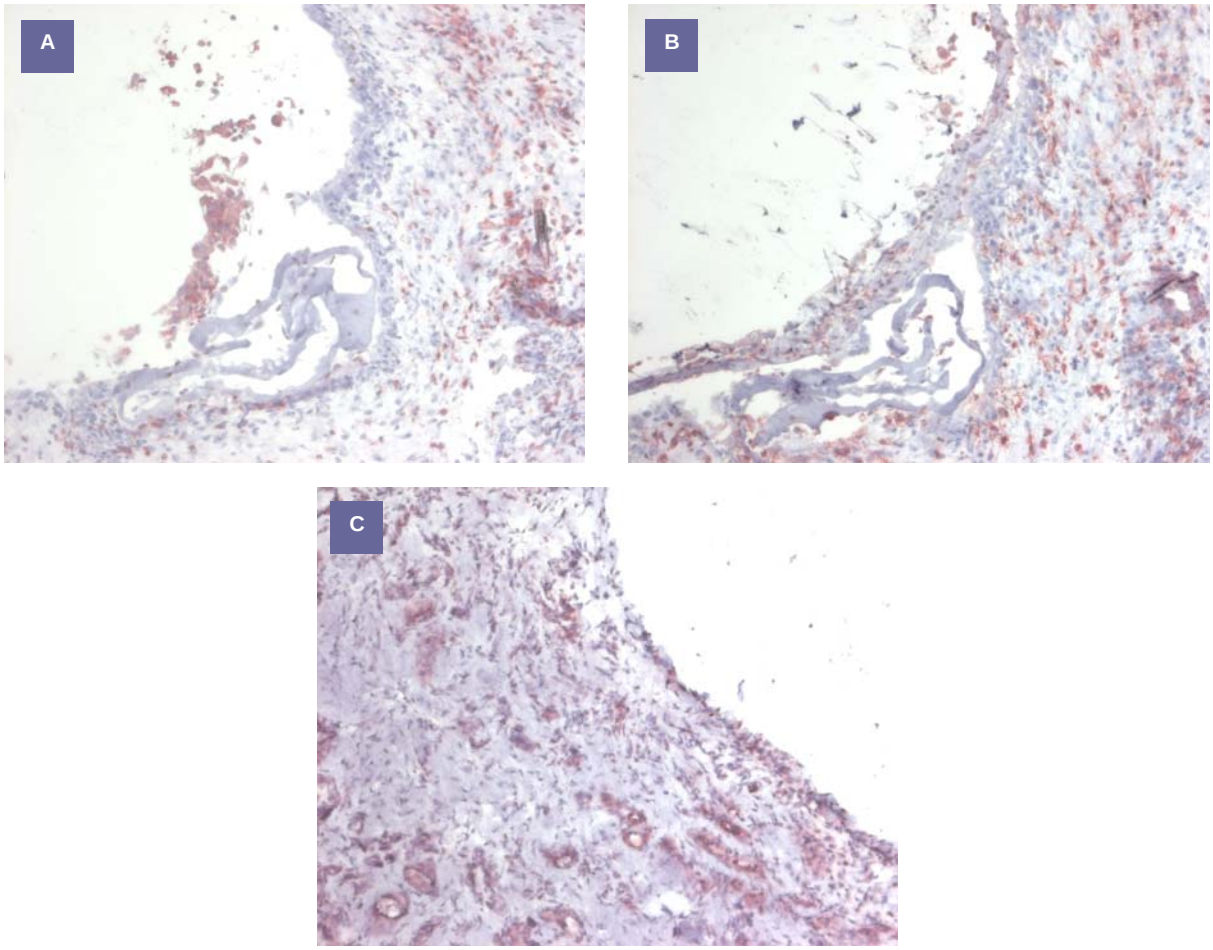

Figure 8.8. Inflammatory response to SPCL. Light micrographs of sections immunocytochemically stained for ED1 (A); MHC II (B); CD54 (C). Explants shown here were taken after (A, B) 7 and (C) 21days. Magnification (x10)

As in the case of the other materials, cells marked with ED1 and ED2 we distributed within the tissue in the close line that describes the material-tissue interface (ED1) and in the outer layer (ED2). Once again, many of the cells expressing MHC II antibodies seem to correspond to cells stained either with ED1 or ED2 therefore suggesting that some of the APC are both sub populations. Compared to the observations for SCA composites, the incorporation of HA in the SPCL polymer seemed to affect the tissue reaction differently. A rare presence of T lymphocytes at the SPCL-tissue interface was noted from day 14. However, in the case of SPCL composites, T lymphocytes were identified at earlier implantation times (7days) and at moderate concentrations in the tissues surrounding SPCL+30% of HA after 21 days of implantation (Fig. 8.9A).

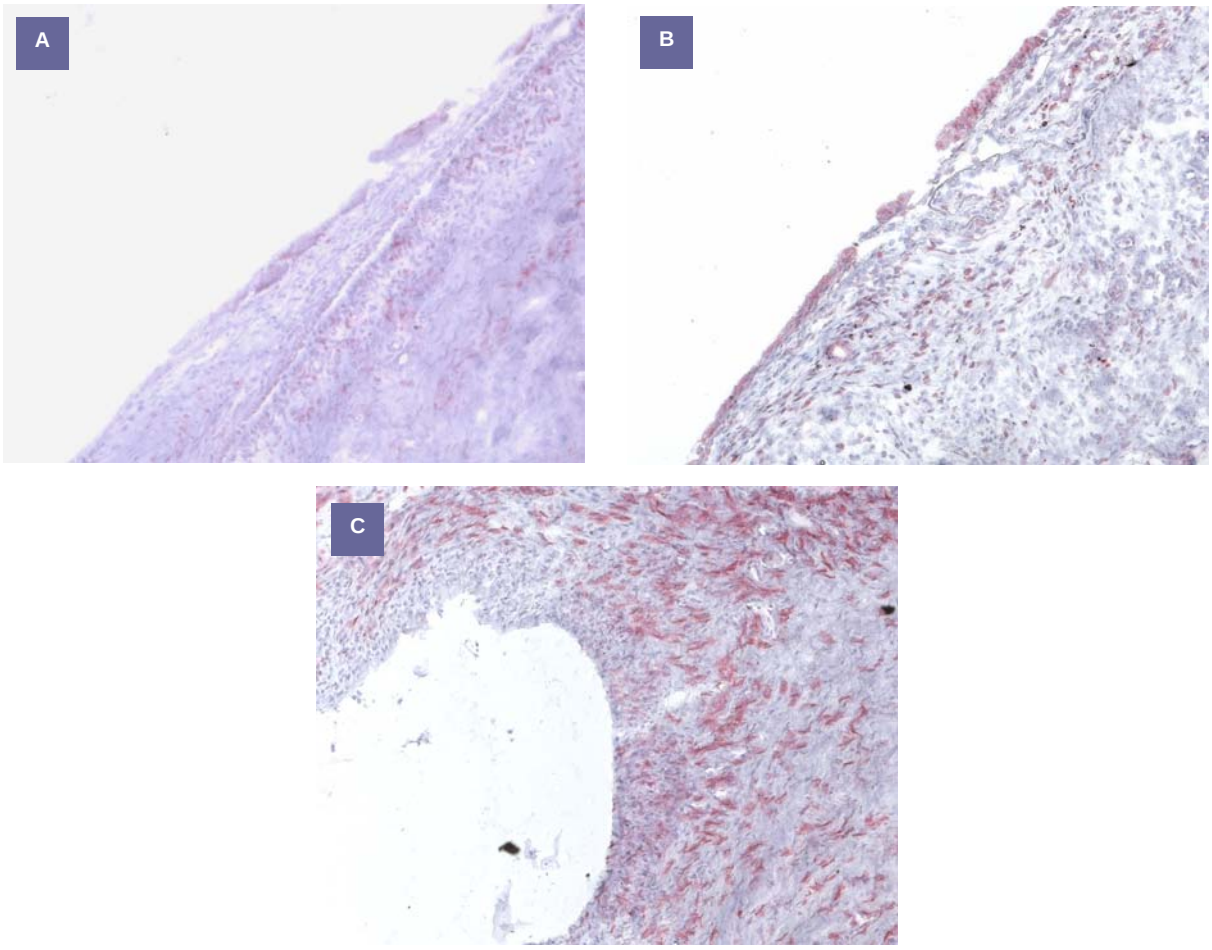

Figure 8.9. Inflammatory response to SPCL composites; (A, B) SPCL+30%HA; (C) SPCL+10%HA. (A) Expression of the α/β T cell antigen receptor in a section immunocytochemically stained for T cells; Light micrographs of sections immunocytochemically stained for ED1 (B); ED2 (C). Explants shown here were taken after (B) 7 and (A, C) 21 days. Magnification (x10)

Considering the ED1 stained cells the implantation of SPCL composites did not attract as many cells as the unreinforced material, although an intense stain seemed to be observed at the SPCL+20%HA interface for shorter implantation periods (Fig. 8.9B). Curiously a very similar pattern between SPCL and its composite reinforced with 20%HA was observed for activated macrophages (CD54 positive). These ICAM-1 expressing cells were abundant from day 7and were observed both at the interface and in the surrounding tissues. At the interface of the other two SPCL composites with 10% and 30% HA, activated macrophages were found in moderate concentrations in the same pattern of distribution.

ED2 positive cells after 21 days of the implantation of SPCL+10%HA presented a different morphology. These positive cells were present, like for the other materials, in the outer layer of the tissue but were bigger (Fig. 8.9C). For all the other composites, ED2 macrophages were comparable in terms of morphology and pattern of distribution.

A higher concentration of cells expressing MHC class II molecules were observed, as for CD54 in the tissues surrounding SPCL+20%HA implant. Similar results were also found for SPCL+10%HA.

8.4. DISCUSSION

The sequence of wound healing processes can be subdivided into two phases: the inflammatory phase, which normally takes about 2 weeks, and the repair phase. The presence of an implant can provide a continuous inflammatory stimulus and as a result the inflammatory phase can be prolonged. This is associated with increased cellular activity and the tissue repair will be delayed and enhanced. Thus, chronic inflammation is characterised by the presence of macrophages, monocytes, lymphocytes and plasma cells with the proliferation of blood vessels and connective tissue. In a final stage, is comprised of foreign body giant cells apposed to the biomaterial surface, surrounded by granulation tissue and fibrous encapsulation of the implant.[44,49-51]

The aim of this study was to determine the presence of the important cell types observed in the tissue response evoked for starch-based biomaterials implanted subcutaneously in rats. The differential activation and expansion of distinct macrophage populations, the recruitment of T cells and the up-regulation of cell-adhesion molecules were evaluated.

It has been suggested that a mild inflammatory reaction to biomaterials includes the formation of a fibrous tissue rich in fibroblasts and with few phagocytic cells[20]. However, the expected response for degradable materials includes a strong inflammatory reaction due to the continued release of the degradation products. Biodegradable starch-based biomaterials did not induce such a severe reaction for the studied implantation times. A comparison between materials with different rates of degradation demonstrated that polymers with faster degradation rates provoked a stronger tissue reaction. The starch-based materials that stimulated stronger tissue responses for the times of implantation studied were SPCL and its composites. These materials were however the polymers with lowest degradation rates, which suggests that this factor was not the most significant factor in the host response to starch-based.

Macrophages stained with ED1, immediately migrated within the first days of implantation and only for some materials their number was increased at longer times of implantation. Thus, the ED1 positive macrophage layer at the implant interface was shown to vary in thickness depending on the material. Mature tissue macrophages (ED2) were only observed in the loose connective tissue surrounding the capsule of the implants and no significant differences were detected with time except for SPCL+10%HA implanted for 21 days.

Some works[9,52-54] have demonstrated varying behaviour and roles for ED1 and ED2 macrophages. ED1 positive macrophages were shown to accumulate quickly and to be active in phagocytosis[9,53], while ED2 macrophages accumulate slowly and play a role in regeneration. One study[55] suggested that ED1 macrophages play a role in material resorption because they mainly act at the material interface. Khouw et al[9] reported that giant cells were never ED2 positive which could suggest that resident macrophages are not involved in the phagocytosis of implanted biomaterials.

Our results for subpopulation distribution are in accordance with those works in terms of recruited macrophages, it was possible to observe the HA reinforcement of SCA induced stronger ED1 staining. We might speculate that within the studied implantation periods there was some HA dissolution from the SCA composites since these are the materials with the higher capacity to uptake water. Thus, macrophages would be recruited to phagocytose these particles.

Nonetheless, the mechanism of cell recruitment and the subpopulations at the inflammation site is still unknown. Some authors[9] question if ED2 macrophages migrate from the loose connective tissue into the biomaterial where they become activated for phagocytosis, loosing their ED2 antigen and turn ED1 positive cells. Others[10] suggest that vascular recruitment of blood born monocytes contributes to the initial macrophage response against the material. In addition, ED2 macrophages capable of express MCP-1[54] were also implied in the stimulation or recruitment of additional macrophages[10]. The duration of the inflammatory reaction has also been correlated with the angiogenesis in the implant [12,13,16,17]. In fact a co-dependence of inflammation and angiogenesis was suggested by some authors[24,25]. Activated macrophages are capable of releasing numerous known angiogenic growth factors.[24,26] The up-regulation of adhesion molecules is known to have a significant participation in the process of transvascular migration of the inflammatory infiltrate[27,28]. Phagocytes adhere to endothelium through ICAM-1, the influx of macrophages was analysed considering the expression of ICAM-1 by macrophages and blood vessel. Angiogenesis varied with the implantation times and also with the materials implanted. A marked vascular response with macrophages infiltrating was observed in the tissues surrounding SCA and SPCL composites especially for higher percentages of HA. However, close to the implants in areas of high cellularity, blood vessels were sparse.

The up-regulation of adhesion molecules is not only useful in the influx evaluation. Cell/cell adhesion predominantly involves binding of ICAM-1 to CD11a or CD11b[56] and macrophages require this interaction to form giant cells. Previous studies found that in interface tissue, ICAM-1 was expressed by giant cells. These multinucleated cells are elemental to phagocytose implanted materials and their degradation products, being found often on the implant side of the membrane but not deeper within the tissue[16,57].

It is believed that macrophage activation and formation of foreign-body giant cells is influenced by the physico-chemical properties of the implant.[18,58] Implants with higher water and carboxylic group content have been shown to inhibit macrophage adhesion and multinucleation, probably because hydrophobic interactions participate in cell-matrix interactions[58].

We found that although for some of the materials an abundant number of activated macrophages, expressing ICAM-1, could be identified, no foreign-body giant cells were present at the implantation site. This observation may be the consequence of the variable rate of degradation of the starch-based material at the time point of the assay, not demanding high phagocytic activity and also of their physical-chemical properties, not appropriated for macrophage adhesion and fusion.

It is known that macrophages have an interactive role with T-helper cells, the activation of T-cells occurs after antigen presentation by the macrophages with the MHC class II molecule[50]. Class II molecules predominantly present antigenic peptides derived from soluble exogenous proteins or extracellular domains of transmembrane proteins mostly to T helper cells. In turn T cells secrete cytokines and provide the necessary signals to promote and regulate humoral and cell-mediated immune responses and inflammation. In particular, activated T-cells may secrete lymphokines like IL-4[32] and IFN-γ[31], two cytokines involved in the regulation of MHC class II molecules and in the formation of FBGC.

This study demonstrated a significant increase in antigen-presenting phenotype at the interface with some materials which can be associated with persistent local chronic inflammation. However, the almost complete lack of lymphocytes may be indicative of an innate mild foreign body reaction against these materials.

8.5 CONCLUSIONS

The *in vivo* observations validated the *in vitro* results confirming that the established *in vitro* models are reliable and can be used to estimate a potential inflammatory reaction provoked by newly developed biomaterials before implantation.

The subcutaneous implantation of starch-based biomaterials in rats allow for concluding that the materials in study possess a weak potential to break out an inflammatory reaction. No macroscopic signs of considerable inflammation were observed and no cellular exudate was formed. SPCL and respective composites were the materials that stimulated the stronger tissue responses but generally biodegradable starch-based materials did not induce a severe reaction for the studied implantation times which contrasts to other types of degradable polymeric biomaterials.

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SECTION IV

MAIN CONCLUSIONS

CHAPTER 9

MAIN CONCLUSIONS

9.1 Cytocompatibility of starch-based biomaterials: polymers and composites

The short-term effect of the degradation products of SEVA-C, SCA and respective composites with 30% of HA reinforcement was evaluated exposing L929 cells to their extracts. The cytotoxicity screening revealed that SEVA-C was the less toxic biomaterial and that its reinforcement with 30% HA induced higher percentage of cell dead due to the leaching of low molecular weight chains formed during processing (thermal degradation) and to the faster degradation rate of the composite.

Despite the less promising results in terms of extract cytotoxicity, when L929 were seeded onto the materials, a slight delaying on cell proliferation was observed in the presence of SEVA-C+30%HA but only for early culture times. The quantification of the number of cells adhered to SEVA-C and SCA did not shown a significant difference comparatively to TCPS. In addition, the cell adhesion behaviour was shown to be determined by surface properties; on SCA, the most hydrophilic and rougher surface, cells presented the typical fibroblast morphology.

Other methodologies were also used to study the cytotoxic potential of starch-based materials with a higher grade of certainty. In addition they also provided the guaranty that if the leachables from the materials interfere with one test system the results are not misinterpreted. It was possible to prove that not only the extract of the materials but also their three-dimensional form has to be biologically tested in order to analyse material-associated parameters that are not possible to consider within the degradation extract.

Therefore, both direct and indirect tests with osteoblast-like cells (SaOs-2) allowed to determine that SCA induced some cytotoxicity and did not present the ideal surface properties for osteoblast-like cells adhesion and proliferation. Contrarily, SPCL extract was not deleterious for cells but did not support their proliferation. Comparatively to the gold standard biodegradable biomaterial (PLLA), SEVA-C and SPCL showed a clearly better behaviour than PLLA in terms of cytotoxicity. The adhesion and proliferation of osteoblast-like cells on SEVA-C and SPLA70 was however, comparable to that of PLLA.

The incorporation of hydroxyapatite had different effects on cytocompatibility according to the polymer matrix studied. In the case of SCA it seemed to change its degradation behaviour and consequently the degradation products released to the culture medium which delayed cell proliferation. In the case of SPCL, the incorporation of HA induced changes in the

surface properties that provoked cell detaching for longer culture times. Different percentages of HA did not seem to change significantly osteoblast-like cell behaviour.

The three blends of starch-based biomaterials induced significantly different adhesion, proliferation and morphology/spreading behaviour of osteoblast-like cells. Depending on the starch-based blend, thus on its synthetic component and the properties that it confers to the surface, cells proliferate at different rates. While SPCL does not support osteoblast-like cells proliferation, SEVA-C and SCA surface properties are respectively the most and the less appropriated for SaOs-2 adhesion and proliferation.

Overall results indicate that starch-based biomaterials present characteristics of cell adhesion/spreading and proliferation that are not disappointing considering their degradable nature, that are in general better that what is observed for PLA based systems. However it must be stressed that different cell types might have dissimilar behaviour on the same surface.

9.2 Immunocompatibility of starch-based biomaterials: polymers and composites

The *in vitro* evaluation of the immunocompatibility of starch-based polymers and composites was focused on changes in the free radical and degranulation activity of neutrophils as well as on their potential to activate immune system cells *in vitro*.

The amount of lysozyme secreted by neutrophils in contact with starch-based polymers and composites was less than 20% of the potential maximum and not significantly dependent on the material except for some SPCL composites. In fact, SPCL+30%HA which induced the production of the higher amount of degradative enzyme.

The chemilluminescence experiments showed that in the presence of starch-based polymers and composites the signal produced by activated neutrophils is reduced. The hypothesis that the results obtained would be due to an effect on cell adhesion or due to the presence of antioxidant species that would scavenge the reactive oxygen species, considered so harmful for the tissues, was proved.

Both lysozyme and chemiluminescence assays revealed a low response of the neutrophils when in contact with starch-based polymers and composites, which allows for considering these materials with weak potential to break out an inflammatory response.

An *in vitro* model was established simulating aspects of the *in vivo* inflammatory response to evaluate individual and collective cellular effects resulting from the interaction of the different populations of inflammatory cells with starch based degradable biomaterials.

The adhesion of PMN's to the surface of starch-based biomaterials was found to be dependent on the blend and on the time of culture. In the presence of ceramic this different affinities also vary according to the unreinforced matrix.

Distinct functional subsets of macrophages together with lymphocytes were found to be adhered to the surface of starch-based polymers and composites. While SCA promoted higher PMN adhesion and lower activation, the number of cells from a mixed population of monocytes/macrophages and lymphocytes was found to be lower on that material, which also showed a reduced amount of activated macrophages.

In terms of inflammatory response, the hydroxyapatite incorporation resulted in low monocyte/macrophage adhesion and in a less potential to activate the cells.

In addition starch-based polymers and composites did not elicit a very strong reaction from immune system cells *in vitro*, demonstrated by a lower production of cytokines when compared to a control PLLA biodegradable material.

There were differences in the amount of released cytokines with respect to culture time, the amount decreasing with increasing culture time. IL-6 was detected in the highest amounts followed by TNF-α. IL-1β was produced in lower amounts and was undetectable in the presence of some of the starch-based materials. No IL-2 or IFN-γ was produced at any of the tested times of culture in the presence of any of the materials.

In general HA reinforcement resulted in lower cell activation, particularly in the case of SEVA-C and SCA potentially enabling better mechanical properties to be combined with reduced levels of cytokine production. On the contrary, SPCL composites did not have a significant effect on cytokine production comparatively to unreinforced SPCL except for TNF-α, which was highly produced.

The subcutaneous implantation in rats allow for concluding that the materials in study possess a weak potential to break out an inflammatory reaction. No macroscopic signs of considerable inflammation were observed and no cellular exudate was formed. Although there were some materials (SPCL and composites) that stimulated stronger tissue responses, all the materials were surrounded by a thin fibrous capsule.

The *in vivo* observations validated the *in vitro* results confirming that the established *in vitro* models are reliable and can be used to estimate a potential inflammatory reaction provoked by newly developed biomaterials before implantation.

The inflammatory response to biomaterials was demonstrated to be a very complex process, certainly influenced by the chemical and physical properties of the materials. These factors did not necessarily act independently and also affected the diverse components of the biological system in different ways.

In general, the cytocompatibility and immunocompatibility studies showed that starch-based polymers and composites are promising biomaterials. Comparatively to the currently used biodegradables, they possess properties that induce similar to better cytotoxicity behaviour. The adhesion and proliferation of osteoblast-like cells on some of these materials was also comparable to that of PLLA which demonstrates their potential to be used in orthopaedic applications. The *in vitro and in vivo* immunocompatibility remarks further support the suitability of starch-based biomaterials to be used in biomedical applications due to their weak potential to break out an inflammatory reaction.