“É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.”
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With the increase in life expectancy, there is a demand to meet new challenges in terms of health care. To the growing awareness of the need of preventive medicine, is allied the regenerative medicine, overcoming the field of replacement or repairing medicine.

The advent of tissue engineering, the science of repair, has created a vast field for research both in terms of materials, as well as new biological approaches that, in combination, create hybrid materials that are expected to achieve the regeneration of the damaged organs/tissues.

Within the tissue engineering field, bone is considered, together with cartilage, as one of the tissues that posses more difficulties for regeneration. Many researchers have pursued, so far without success, the creation of the ideal scaffold that could meet all the though orthopaedic demands. Another important aspect of bone tissue engineering has to do with the role of key players such as cells and growth factors. A hybrid approach, combining a scaffold material, progenitor cells and growth factors seems to hold a great potential, which has been studied by several researchers. Based on this approach, it is necessary to develop materials that can fulfill all the requirements – provide structural support, serve as carriers for cells and act as drug delivery systems – creating a material that can be a “3-in-1” system.

The objectives of the present work were to develop new systems that can fulfill the requirements referred above. Microparticles based on starch-based materials were chosen, as these materials have shown potential for biomedical applications. The developed materials were evaluated in terms of their eventual bioactivity, non-cytotoxicity, ability to serve as carriers for cell adhesion and proliferation, as well as their potential to be used as controlled delivery systems either for anti-inflammatory agents or growth factors.

Several starch-based materials were used for the development of polymer-based microparticles, and among them, two materials were selected for further studies: a blend of starch and polylactic acid (SPLA, 50:50 wt) and a chemically modified potato starch, Paselli 2. Both materials enabled the synthesis of particulate systems, both polymer and composite [with Bioactive Glass 45S5 (BG 45S5)].

The in vitro bioactivity of the materials, denoted by the ability of the materials to form a calcium-phosphate layer at their surface when immersed in a solution simulating the blood plasma ionic composition, was confirmed for SPLA-30%BG composites and for both polymer (Pa2) and composite (Pa2-30%BG) particles.

The cytotoxicity was evaluated by using leachables of the materials, through MEM, MTT and total protein quantification. All the materials were proved to be non-toxic. The tested materials were shown to be able to support cell adhesion and proliferation throughout the culture period, while allowing the maintenance of the osteoblastic phenotype of the cells, as confirmed by RT-PCR.

The developed systems were also characterized regarding their ability to be used as release systems. Two categories of bioactive agents - corticosteroids and growth factors – were incorporated within the matrix of SPLA particles. Dexamethasone and two other corticosteroids with a similar structure were chosen, the former as a differentiation agent for cells of the osteoblastic lineage and the latter as anti-inflammatory
agents. Platelet-Derived Growth Factor (PDGF) was selected by its mitogenic effect over cells from the osteoblastic lineage, therefore adequate for the stimulation of cell proliferation.

The loading efficiency reached values of 80% and 17% for corticosteroids and PDGF, respectively, and these are similar to the ones observed for other systems. The release profiles have shown a slow, sustained release for corticosteroids and a burst release in the initial stage for PDGF. For the latter, the low loading efficiency did not hinder the ability of this system to be used in an in vitro strategy where cell expansion is to be achieved.

The mitogenic effect of PDGF after release was tested using cultures of osteoblast-like cells, which markedly showed that the incorporation and release of PDGF from starch-based microparticles does not affect its biological activity (i.e., mitogenic effect).

In summary, the results of this work show that it was possible to develop starch-based microparticles, whose characterization showed that they are fabricated with controlled sizes, they are biodegradable, non-cytotoxic and bioactive, when immersed in a simulated body fluid. Additionally, the developed materials were shown to be able to incorporate and to release with a defined profile molecules pertaining to two different groups of bioactive agents (corticosteroids and growth factors) and to allow cell adhesion, proliferation and expression of the osteoblastic phenotype of primary cells and of a cell line.

The results herein presented demonstrate the potential of the developed systems for application in the tissue engineering field, namely:

- As possible scaffold/filling materials for applications in bone defects were non-load bearing materials are desirable;
- As substrates/carriers for the culture and expansion of cells in 3-D bioreactors;
- As release systems for growth factors and other biologically active molecules;
- As components of a three-fold strategy incorporating the above-named points – structural support, carriers for cells and as systems for the release of biologically active agents.
**Micropartículas para Liberação Controlada de Fármacos e Metodologias Inovadoras em Engenharia de Tecidos**

**RESUMO**

Com o aumento da esperança média de vida para cerca de 80 anos há necessidade de responder a novos desafios em termos de cuidados de saúde. À medicina preventiva alia-se a medicina regenerativa, em detrimento da medicina simplesmente “reparadora”.

O advento da ciência da regeneração, a Engenharia de Tecidos, criou um vasto campo de investigação, quer em termos de novos materiais e materiais modificados que possam funcionar como suportes, quer em termos de novas abordagens no campo biológico que, quando combinados, tem o potencial de criar materiais híbridos que se espera venham a promover a regeneração e reparação de tecidos danificados.

No campo da Engenharia de Tecidos, o osso é considerado, juntamente com a cartilagem, como um dos tecidos mais difíceis de regenerar. Muitos grupos de investigação tentam, há bastante tempo, através de várias abordagens, criar o suporte ideal que possa corresponder a todos os requisitos necessários para regenerar um tecido ósseo. Outro aspecto chave na engenharia de tecidos do osso diz respeito aos sistemas biológicos, especificamente as células ósseas e os factores de crescimento. A combinação de um material de suporte com células detentoras de potencial proliferativo e factores de crescimento constitui uma estratégia promissora neste domínio, que tem vindo a ser estudada por diversos investigadores. Com base nesta abordagem, é necessário o desenvolvimento de materiais que possam servir estas três funções – suporte estrutural, suporte para adesão e proliferação de células e como sistemas de libertação controlada de fármacos - criando um material que seja “3-em-1”.

Os objectivos do presente trabalho visaram o desenvolvimento de novos sistemas que possam preencher os requisitos referidos acima. A escolha de materiais sob a forma de micropartículas, usando materiais à base de amido, apresenta um grande potencial para este tipo de abordagem. Os materiais desenvolvidos foram caracterizados em relação à sua eventual bioactividade, não citotoxicidade e capacidade de serem usados como veículos para a adesão e proliferação de células ósseas, assim como o seu potencial como sistemas de libertação de agentes anti-inflamatórios e factores de crescimento.

Para o desenvolvimento das micropartículas foram usados, como matéria prima, vários materiais à base de amido. Posteriormente foram selecionados, com base em vários critérios, dois materiais: uma mistura de amido e poliácido láctico (SPLA, 50:50 peso) e um amido de batata modificado quimicamente de modo a ser solúvel em água (Paselli 2). Ambos os materiais demonstraram capacidade de formar micropartículas, quer poliméricas, quer compósitas, reforçadas com vidro bioactivo 45S5 (BG 45S5). A bioactividade *in vitro*, avaliada pela capacidade dos materiais de formar ou não à superfície uma camada de fosfato de cálcio, quando imersas numa solução que simula a composição iônica do plasma sanguíneo, foi confirmada para as partículas compósitas de SPLA-30%BG e para as partículas poliméricas e compósitas de Pa2 e Pa2-30%BG, respectivamente.
A eventual citotoxicidade dos materiais desenvolvidos foi avaliada com recurso a materiais “lixiviados” das partículas, através de testes MEM, MTT e quantificação da proteína total. Os resultados confirmam a ausência de toxicidade dos materiais. Todos os materiais testados mostraram ser capazes de permitir a adesão e proliferação das células in vitro, permitindo simultaneamente a manutenção do fenótipo osteoblástico das mesmas, como confirmado por RT-PCR e por coloração com Alizarin Red.

Os sistemas desenvolvidos foram também caracterizados quanto à sua capacidade de serem usados como sistemas de libertação controlada de moléculas bioactivas. Dois representantes de moléculas bioactivas - corticosteróides e factores de crescimento - foram incorporados nas partículas de SPLA. A escolha recaiu na Dexametasona e dois outros corticosteroides, pelo papel desempenhado por estes factores como potentes anti-inflamatórios e no caso da Dexametasona como indutor da diferenciação de células na linhagem osteoblástica. De entre os factores de crescimento com efeito documentado no tecido ósseo a escolha recaiu no Platelet-Derived Growth Factor (PDGF), que tem um efeito mitogénico sobre osteoblastos e, como tal, será adequado para estimular a expansão celular. A eficiência de incorporação atingiu valores de cerca de 80% e 17% para os corticosteróides e para o PDGF, respectivamente, semelhantes aos observados para outros sistemas. Os perfis de libertação revelaram uma libertação lenta dos corticosteróides e rápida, nos estados iniciais, para o factor de crescimento. Para o PDGF, o baixo valor de incorporação observado não impediu, no entanto, de observar in vitro um perfil de libertação desejável para expansão da população celular. A capacidade mitogénica do PDGF após incorporação e libertação foi avaliada com recurso a culturas de células osteoblásticas, que mostrou que células estimuladas com PDGF libertado das micropartículas proliferaram a uma taxa superior às células não estimuladas.

Em suma, os resultados deste trabalho mostram que foi possível desenvolver micropartículas à base de amido, cuja caracterização revelou que podem ser produzidas com tamanhos definidos, são biodegradáveis, não são citotóxicas e são bioactivas quando imersas numa solução que simula a composição iónica do plasma sanguíneo. Adicionalmente, os materiais desenvolvidos demonstraram possuir a capacidade de incorporar e libertar, com um perfil definido, moléculas pertencentes a dois grupos distintos (corticosteróides e factores de crescimento) e permitirem a adesão, proliferação, assim como a expressão do fenótipo osteoblástico de culturas primárias e de uma linha celular.

Os resultados aqui apresentados demonstram o potencial dos sistemas desenvolvidos para aplicação em engenharia de tecidos, nomeadamente:
- Como possíveis materiais de suporte para preenchimento de defeitos ósseos em aplicações que não exigam elevadas propriedades mecânicas;
- Como suportes para a cultura e expansão de células em bioreactores tridimensionais;
- Como sistemas de libertação controlada de fármacos e factores de crescimento.
- Como componentes de uma estratégia de engenharia de tecidos, combinando num só material propriedades como suporte estrutural, suportes para a cultura de células e como sistemas de libertação controlada de fármacos.
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<tr>
<td>ACS</td>
<td>American Chemical Society</td>
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<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>AR</td>
<td>Alizarin Red</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>BBT</td>
<td>2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole</td>
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<tr>
<td>BBTP</td>
<td>2′-[2-benzothiazoyl]-6′-hydroxybezothiazole phosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchorinic acid</td>
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<tr>
<td>BCP</td>
<td>Bi-calcium Phosphate</td>
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<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic factor</td>
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<tr>
<td>BG</td>
<td>Bioactive Glass</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BSE</td>
<td>Bovine Spongiform Encephalopathy</td>
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<tr>
<td>Ca-P</td>
<td>Calcium Phosphate</td>
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<tr>
<td>CB</td>
<td>Chondroblast</td>
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<tr>
<td>CJD</td>
<td>Creutzfeldt Jakob Disease</td>
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<tr>
<td>CMFDA</td>
<td>5-chloromethylfluorescein-di-acetate</td>
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<td>Col Iα</td>
<td>Collagen Iα</td>
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<tr>
<td>DDSs</td>
<td>Drug Delivery Systems</td>
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<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<tr>
<td>DNA</td>
<td>Desoxyribonucleic Acid</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
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<tr>
<td>ECAAC</td>
<td>European Collection of Cell Cultures</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDS</td>
<td>Energy Dispersive Spectroscopy</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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ELISA - Enzyme Linked Immunosorbent Assay
EPR - Enhanced Permeation & Retention

F
FBS - Fetal Bovine Serum
bFGF - basic Fibroblast Growth Factor
FTIR- ATR - Fourier Transformed Infrared Spectroscopy – Attenuated Total Reflectance

G
GADPH - Glyceraldehyde phosphate hydrogenase
GF - Growth Factor
GFP - Green Fluorescence Protein

H
HA - Hydroxylapatite
HARV - High Aspect Ratio Vessel
HGF - Hepatocyte Growth Factor
HIV - Human Immunodeficiency Virus
HPLC - High Performance Liquid Chromatography
HSA - Human Serum Albumin

I
ICP-OES - Inductively Coupled Plasma – Optical Emission Spectroscopy
IFN - Interferon
IGF - Insulin-like Growth Factor
rIGF - recombinant Insulin-like Growth Factor
IgG - Immunoglobulin G
IL – Interleukin
ISO - International Standards Organization

K
Kd - KiloDalton

M
MAP - Mitogen Activated Protein
MBG – Modified Bioactive Glass
MEM - Minimal Essential Medium
MP - 16-α-Methylprednisolone
MPA - 16-α-Methylprednisolone acetate
MSCs - Mesenchymal Stem Cells
µg - Micrograms
µl - Microliters
µm - Micrometers
mg - Miligrams
ml - Milliliters
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide
Mw - Molecular weight

N
NASA – National Aeronautics and Space Administration
NGF - Nerve Growth Factor
ng - Nanograms

O
OB - Osteoblasts
OC - Osteoclasts
OCN - Osteocalcin
OP - Osteogenic Protein
OPF - Oligopropylene fumarate
OSP - Osteopontin

P
Pa2 - Paselli 2
PAA - Poly(acrylic acid)
PAM - Pharmacologically active microcarriers
PBLG - Poly(γ-benzyl L-glutamate)
PBS - Phosphate Buffered Solution
PBS-AB - Phosphate Buffered Solution with antibiotics
ε-PCL - ε-Polycaprolactone
PDGF - Platelet Derived Growth Factor
pE2 – Prostaglandin E2
PEG - Polyethylene Glycol
PEO - Poly(ethylene oxide)
PGA - Polylglycolic acid
PHBV - Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PIBCA - Poly-(isobutylcyanoacrylate)
PIHCA - Polyisohexylcyanoacrylate
PLA - Polylactic acid
PLGA - Polylactic-co-glycolic acid
PMMA - Polymethyl methacrylate
pNP - p-Nitrophenol
pNPP - p-Nitrophenyl Phosphate
POE – Polyoctoesthers
PRP - Platelet-Rich Plasma
PTC - Poly (trimethylene carbonate)
PTH - Parathyroid hormone
PVA - Polyvinyl alcohol
PVP - Polyvinylpyrrolidone

R
RBMCs - Rat Bone Marrow Cells
rpm – rotations per minute
RT-PCR - Rerverse Transcriptase – Polymerase Chain Reaction
RWV – Rotating Wall Vessel

S
SCA - Starch Cellulose Acetate
SEM - Scanning Electron Microscopy
SEVA-C - Starch-Ethylene Vinyl Alcohol copolymer
SPLA - Starch-Poly(lactic Acid)

T
3-D - Three-dimensional
TCP - Tricalcium Phosphate
TCPS - Tissue Culture Polystyrene
TE - Tissue Engineering
TGF - Transforming Growth Factor
TNF - Tumor Necrosis Factor
TSTP - Trisodium trimethaphostate

U
UV - Ultraviolet

V
VEGF - Vascular Endothelial Growth Factor

W
Wt - Weight

X
XRD - X-Ray Diffraction
Chapter I
MATERIALS IN PARTICULATE FORM FOR TISSUE ENGINEERING.
BASIC CONCEPTS & APPLICATIONS IN BONE

**Figure 1.** Schematics of release of entrapped bioactive agents from biodegradable polymeric particles. When the polymer device incorporating the active agent (A) is inserted into the environment, the fluid from the surrounding medium enters the matrix (B), causing swelling of the device (C). The fluid creates diffusion channels (C) and the incorporated active agent is released to the external environment (D). In the case of biodegradable polymers, the device removal will occur by the degradation of the material.

**Figure 2.** Release profile for biodegradable polymers. The first stage (1) is a burst release, caused by diffusion of the bioactive agent located closer to the surface. The second stage (2) is caused by gradual degradation of the polymer and the third stage (3) is characterized by massive degradation (solubilization) of the material.

**Figure 3.** Healing process of bone, depicted in a simplified diagram. After the defect is created, there is the formation of a blood clot. Apart from the formation of a haematoma, haematopoietic precursors (H) in the bone marrow differentiate into osteoclasts (OC) that start the process of resorbing the end bone of the defect. Mesenchymal cells (MSCs) within the bone marrow are stimulated to migrate to the healing site. These cells originate chondrogenic cells (CB) that produce an intermediate cartilaginous matrix that progressively mineralizes. This cartilaginous phase is then replaced by new bone synthesized by osteoblasts (OB). Not depicted is the role of vascularization.

SECTION 3

Chapter III
MICROPARTICULATE RELEASE SYSTEMS BASED ON NATURAL ORIGIN MATERIALS

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Figure 3. Water uptake (A) and weight loss (B) profiles for SPLA and SPLA/BG particles. Composite particles have a similar water uptake profile as SPLA particles, differing in the weight loss, which is lower than the one observed for the polymeric particles.

Figure 4. Scanning electron microscopy images representative of Pa2 particles (A, B). Regarding the size distribution, particle size ranges from few microns (A) to hundreds of microns (B). Morphological evaluation shows that the particles seem to be composed of smaller particles, all brought together. This feature creates pores in the particles, but they do possess a dense matrix with only very small pore sizes.

Figure 5. Scanning electron microscopy images representative of Pa2-BG composite particles (A, B). Size range is the same as compared with polymeric particles, however differences in the morphology do occur. Composite particles are more regularly spherical, with a smoother surface. In Pa-BG particles the cross-section of a composite particle cut to expose its interior shows the BG 45S5 granules dispersed in the starch network.

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Chapter V
SOLUBLE STARCH AND COMPOSITE STARCH-BG 45S PARTICLES: SYNTHESIS, BIOACTIVITY AND INTERACTION WITH RAT BONE MARROW CELLS

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SECTION 4

Chapter VI
THE EFFECT OF STARCH AND STARCH – BIOACTIVE GLASS COMPOSITE MICROPARTICLES ON THE ADHESION AND EXPRESSION OF THE OSTEOBLASTIC PHENOTYPE OF A BONE CELL LINE

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**Chapter VII**

**ENTRAPMENT ABILITY AND RELEASE PROFILE OF CORTICOSTEROIDS FROM STARCH-BASED PARTICLES**

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Chapter VIII
STARCH-BASED MICROPARTICLES AS CARRIERS FOR THE RELEASE OF ACTIVE PLATELET-DERIVED GROWTH FACTOR

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Cartoon illustrating the strategy proposed for generating hybrid (bioactive molecule-scaffold-cell) constructs. The polymer and growth factor (GF) are combined to form a scaffold microparticle entrapping the growth factor within its matrix (1.). These microparticles are then combined with appropriate cells (progenitor or lineage specific) (2.) and cultured for an appropriate time period (3.). During culture, the growth factor incorporated in the microparticles is released by diffusion and by degradation of the scaffold matrix (4.), thus stimulating the adhered cells to proliferate, differentiate and form mineralized matrix (5.). Ultimately, the scaffold totally degrades and is completely replaced by new tissue.
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Gabriela A. Silva was born in 1978 in Arcos de Valdevez, Portugal. She presently lives in Braga and works, as a researcher, in the 3B’s Research Group (Biomaterials, Biodegradables and Biomimetics), under the supervision of Rui L. Reis.

Her background includes a four-year graduation in Applied Biology, by the School of Sciences, University of Minho. She has just submitted her PhD thesis on Materials Science and Technology – Tissue Engineering/Hybrid Materials to the University of Minho, which was prepared in cooperation with the University of Pennsylvania and the Thomas Jefferson University, both in Philadelphia, USA.

During the last year of her graduation, Gabriela A. Silva worked in the animal cell field, her work focusing on the *in vitro* culture and regenerative potential of isolated hair follicles, under a grant of the Portuguese Health Ministry. This work was the subject of her senior research project.

Parallel to her research within this project, she completed with success two post-graduation courses, one on Forensic Sciences (Instituto de Medicina Legal do Porto) and another on Molecular Genetics (School of Sciences, University of Minho). She was also the lab instructor of a course (Biochemistry and Cell Physiology) of the major in Optometry, at the University of Minho.

In 2001 she joined the 3B’s Research Group, where she has been working ever since on the development of new microparticle systems for both controlled release and tissue engineering applications. During this phase she worked for different time periods at the University of Pennsylvania, under supervision of Professor Paul Ducheyne and at the Thomas Jefferson University, under supervision of Professor Irving M. Shapiro, under the same scope of study.

As a researcher of the 3B’s Research Group she has been involved in the supervision of senior research projects of undergraduate students, as well as in the organization of two NATO/ASI meetings, one in Tissue Engineering (2001) and another in Biomineralization (2003), both held in Alvor, Portugal.

Gabriela A. Silva has also been involved in the preparation of several grant proposals, both at the National (Portuguese Foundation for Science and Technology) and European levels (frameworks V and VI), including the only Tissue Engineering Network of Excellence of Europe, EXPERTISSUES, as well as other EU-funded projects, such as HIPPOCRATES and also PROTEUS, a transnational project between Portugal and Spain.

As a result of her research work she attended the most important international meetings in the present field of research. Presently she is the author of 11 papers in international refereed journals (7 published, 2 in press, 1 accepted, 1 submitted), 3 book chapters (3 published) and 13 abstracts published in international conference proceedings.
This thesis is based on the following publications:

**International Journals with referee**


GA Silva, OP Coutinho, P Ducheyne, IM Shapiro, RL Reis, *The effect of starch and starch – bioactive glass composite microparticles on the adhesion and expression of the osteoblastic phenotype of a bone cell line*, 2006, Biomaterials, *accepted for publication*.

Book Chapters


Communications in international meetings


GA Silva, ACP Dias, OP Coutinho, RL Reis, *Evaluation of the Encapsulation Efficiency and Release Profile of Two Methylated Forms of a Corticosteroid Model Drug on/from Novel Starch-Based Microparticles*, 18th European Society for Biomaterials (ESB2003), Stutgard, Germany, October 2003.

FJ Costa, GA Silva, NM Neves, OP Coutinho, RL Reis *Synthesis, Characterization and Bioactivity of Starch-Based Microparticles for Biomedical Applications* NATO/ASI course – Learning from nature how to design new implantable biomaterials: from biomineralization fundamentals to biomimetic materials and processing routes Alvor, Portugal, October 2003.

GA Silva, FJ Costa, OP Coutinho, RL Reis, *Response of Primary Rat Bone Marrow Cells to Novel Starch-Based Microparticles aimed to be used as drug delivery carriers* NATO/ASI course – Learning from nature how to design new implantable biomaterials: from biomineralization fundamentals to biomimetic materials and processing routes Alvor, Portugal, October 2003.
GA Silva, FJ Costa, OP Coutinho, RL Reis, *The Response of Rat Bone Marrow Cells Cultured With or Without Dexamethasone to Starch-Based Microparticle Drug Delivery Carriers, 4th World Biomaterials Congress* (WBC), Sidney, Australia, May 2004.


This thesis is divided in five sections containing nine chapters, with six of them experimental. The contents of each are summarized bellow.

Section 1 (Chapter I) gives an overview of the state of the art of the role played by materials in the particulate form in tissue engineering applications. The first part is focused on the specifics of those systems, namely on the materials and methods of production and major applications. The second part relates to their applications in and potential for bone tissue engineering.

Section 2 (Chapter II) contains in detail the materials and experimental procedures used for the development and characterization of starch-based microparticles.

Section 3 (Chapters III, IV and V) describes the development and characterization of starch-based microparticles, using several raw materials. Chapter III presents an overview of the developed microparticles, from which were chosen the materials to be further studied. Chapters IV and V describe the synthesis of starch-based and starch-based/Bioactive Glass 4S5 particles and their characterization in terms of bioactivity, eventual cytotoxicity and the response of rat bone marrow cells to the developed systems.

Section 4 (Chapters VI, VII and VIII) is composed of studies performed to assess potential applications of the above-described systems, namely their use as carriers for bioactive agents and substrates for cells for innovative tissue engineering applications. Chapter VI describes the interaction of the developed systems with cell populations typical from an eventual target tissue. For this purpose we have studied the adhesion, proliferation and maintenance of the osteoblastic phenotype of mouse calvaria osteoblasts when cultured at the surface of starch-based microparticles.

To further assess the potential of the developed starch-based systems to be used as carriers for bioactive molecules, Chapters VII and VIII contain data regarding the release of two different groups of bioactive molecules: in chapter VII, the bioactive agents were corticosteroids, and in chapter VIII, the chosen bioactive agent was Platelet-Derived Growth Factor (PDGF). Both types of bioactive agents were incorporated within the matrix of starch-based microparticles, and could be released in a sustained manner over time, maintaining their biological activity.

Finally, section 5 (Chapter IX) contains the general conclusions regarding the overall work carried out under the scope of this thesis, as well as some final remarks.
PART A. BASIC CONCEPTS ON MATERIALS IN PARTICULATE FORM

Abstract

For biomedical applications, materials so small in size are growing in importance. In an era where nano is the new trend, micro and nano materials are in the forefront of developments.

Materials in the particulate form aim to designate systems with a reduced size, such as micro and nanoparticles. These systems can be produced starting from a diversity of materials, of which polymers are the most used ones. Similarly to the materials used, so is the multitude of methods to produce particulate systems, and both materials and methods are critically revised herein.

Among the varied applications that materials in the particulate form can have, drug delivery systems is probably the most prominent one, as these have been in the forefront line of interest for biomedical applications. The basic concepts pertaining to drug delivery are summarized, and the role of polymers as drug delivery systems wrap up this review.

*This part of the chapter is based on the following publication:
1. DEFINITION

The key feature of particle materials system being their reduced size, the question regarding threshold size for considering a system as being a particulate one is of value. Across literature, many authors differ regarding the threshold size to which a system can be classified within this category. Herein, micron size systems in the range from 1 to 1000 micrometers (\(\mu m\)), will be considered first. Nano size particle systems within this context are those for which the sizes are below 1 \(\mu m\), and will be described following micron size systems.

2. CLASSIFICATION OF MATERIALS IN PARTICULATE FORM

2.1. Microparticles

*Microparticles* consist of particles in a size range of 1 to 1000 \(\mu m\). These include microcapsules, vesicular systems in which a cavity is surrounded by an unique polymeric membrane and microspheres, which are matrix-filled systems. Polymer microspheres have attracted attention as carrier matrices in a wide variety of medical and biological applications, such as affinity chromatography, immobilization, immunoassay, nuclear imaging and cell culturing. Additionally, the incorporation of bioactive agents into small polymeric particles was recognized years ago by the pharmaceutical industry as a viable means of improving drug delivery. This use arose because conventional dosage forms, such as oral delivery and injection were not able to control the rate of delivery or the target area of the bioactive agent and were often associated with an immediate or rapid release.

The main advantages of microparticles is that they may be administered by injection or intranasally as a dry powder, so that a surgical procedure is not required, and that they may
contain a greater amount of biologically active molecule per unit volume\textsuperscript{14-16}. Various parameters including particle size and distribution, porosity, pore structure and surface area are considered to describe the overall performance of polymer microparticles in biomedical applications\textsuperscript{4,17,18}. Additionally, the use of microparticles composed of biodegradable polymers eliminates the need for device removal after release of the agent\textsuperscript{11}. Based on these features, microparticles have been the subject of numerous studies with the intent to overcome a number of issues related to therapeutics of biologically active molecules.

In summary, microparticles have the following properties that render them attractive:

- **Size**: small size allows them to be inserted in the target area in a non-invasive manner, thus increasing effectiveness;

- **Size distribution**: microparticles ranging from a few to a few hundred microns can be selected according to a specific application;

- **Porosity and pore structure**: the presence of pores allows the tailoring of the release profile and

- **Surface area**: large surface area and capacity of loading the bioactive agent at a high fraction of the total weight of the particle.

However, for some applications, particles with an even smaller size – nanoparticles – can be desirable over microparticles.

### 2.2. Nanoparticles

Nanoparticles, being submicron systems, have the advantage of an even larger surface area compared with microparticles, because the total surface area is inversely proportional to the third power of the diameter\textsuperscript{19,20}. In these systems the bioactive agent can be dissolved, entrapped, encapsulated, adsorbed, immobilized or attached to the matrix\textsuperscript{21}, and depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained\textsuperscript{2,22}. Nanocapsules
are vesicular systems in which the bioactive agent is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the bioactive agent is physically and uniformly dispersed\textsuperscript{22}. Nanospheres and nanocapsules are the morphological equivalents of microspheres and microcapsules, respectively\textsuperscript{17}.

Nanoparticles can be injected and as a result, can circulate in the blood stream\textsuperscript{23}. However, in some cases, nanoparticles are phagocytized by macrophages\textsuperscript{24}, and this can lead to an adverse immunological response. However, such reaction may be desirable in applications such as vaccination therapies and when enhanced uptake of exogenous compounds, such as anti-HIV (Human Immunodeficiency Virus) drugs\textsuperscript{24}, is sought. Nanoparticle polymeric carriers, when their size is less than 100 nm, have a high potential for being accumulated in tumor sites by the enhanced permeation and retention (EPR) effect\textsuperscript{25,26}. Hydrophilic modification, particularly by introducing poly(ethylene)glycol (PEG) by physical coating or covalent linking – a process denominated pegylation - to the surface, prolongs the half-life of the carriers\textsuperscript{27-29} during circulation in blood by reducing opsonization and thus minimizing carrier clearance in organs such as liver, spleen, lung and bone marrow\textsuperscript{30,31}. This long-circulating stealth characteristic of the carrier produces the EPR effect which is valuable in passive cancer targeting\textsuperscript{32,33}.

Nanoparticles hold great potential for the treatment of tumors. An example is related to the ability of those materials to include within their matrix magnetic particles and by directing nanoparticles to the target (e.g. tumor cells) through magnetic fields created around the tumor. This brings great advantages, such as the reduction of the dosage and side effects, as well as a raise in the therapeutic effect and it is capable of controlled and most importantly direct targeting of the tumor site\textsuperscript{34}.

Nanoparticles offer other specific advantages over liposomes, because they increase the stability of bioactive agents/proteins and possess a better set of controlled release properties\textsuperscript{20,22,29,35-37}. Summarizing, nanoparticles possess the following advantages:

- **Stability**: increased stability over liposomes and promotion of increased stability of entrapped bioactive molecules
- **Surface area**: higher surface area even when compared with microparticles
- **Size**: depending on their size they can be phagocitized or can circulate long enough to promote the therapeutic effect
- **Stealth effect**: controlled by size and modification by coating with polymers such as PEG
- **Delivery to target site**: easily delivered by injection, without the need of invasive procedures.

### 3. OVERVIEW OF SYNTHESIS METHODS

There are several methods for the production of micro and nanoparticles, but the most widely used techniques are methods based in emulsions such as suspension polymerization, solvent evaporation and to a smaller extent, organic phase separation (coacervation) and spray drying methods, as reviewed/described in detail in references 1, 4, 22, 23, 38-40.

In suspension polymerization the monomer phase is broken into droplets (a few microns in diameter) within a dispersion medium (usually an aqueous phase) and stabilized by a surfactant dissolved in the medium. These monomer droplets containing a monomer phase soluble initiator are then individually polymerized by applying a temperature/agitation program. In the emulsion/solvent evaporation method, the polymer is solubilized/dispersed in an organic solvent (for instance, methylene chloride, chloroform) and the resultant solution is then emulsified with an aqueous phase. The formation of the particles is achieved by hardening resulting from the evaporation of the organic solvent. Stirring speed is usually the parameter controlling the size of the particles. This method is easy to implement and yields very good results with a variety of raw materials.

Most of the methods for the production of particle-based systems are actually based on the creation of emulsions between organic and aqueous phases, and suffer one common drawback: the need of organic solvents (such as methylene chloride, chloroform, acetonitrile and...
tetrahydrofuran) in at least one of the production steps\textsuperscript{43-46}. The residual content of the organic solvent in the microparticles after preparation has to be removed in time consuming drying steps\textsuperscript{47}, and in many cases the presence of an organic solvent can lead to loss of the activity of the agent to be loaded into the system. Currently, methods that obviate the use of organic solvents are in demand, and this aspect is particularly critical when there is a risk of hindering the activity of the biological agent. An interesting new approach trying to address this particular issue is the one described by Nykamp \textit{et al.}\textsuperscript{47}, where the authors used a jet milling technique to produce polylactic acid (PLA) and polylactic/glycolic acid (PLGA) microparticles with different ratios of the two polymers. Conceivably, this method could also be used for other polymers. However, the first step of this process involves melting of the starting material, which obviously has to be taken into account when aiming to use the developed systems for delivery of bioactive agents. Similarly, Lin \textit{et al.}\textsuperscript{48} have used a solvent free method to produce polycaprolactone (PCL) microparticles, by dispersing polyethylene glycol (PEG) in the PCL phase. Although the melting temperature of PCL is low (close to 60ºC), this temperature might still be deleterious for the activity of bioactive molecules.

One has to be cautious in choosing the method of production, and weigh carefully between the risks of using an organic solvent or using high temperature conditions, two major parameters influencing the biological activity of an agent.

Although micro and nanoparticles can be produced using a vast array of possibilities, a number of variables that affect the product obtained have to be taken into account when choosing a material and method. These include\textsuperscript{8,9,49}:

- Type and amount of material used;
- Degradation rate of the polymer;
- Type and payload of bioactive agent being incorporated (in case of drug delivery applications);
- Organic solvent being volatilized;
- Type and amount of surfactant dissolved in the aqueous phase;
- Temperature;
- Pressure during solvent evaporation and
- Ratio of the volume of organic solvent/volume of aqueous phase.

By "playing" with the above-referred parameters, researchers have been able to use a wide array of materials and methods for a number of applications.

4. MATERIALS USED IN THE SYNTHESIS OF MATERIALS IN PARTICULATE FORM

The polymeric class of materials has been regarded as the primary choice for applications where small size particles are needed, since many polymers can be formed into microparticles and nanoparticles for delivery and other applications. These may be non-degradable or degradable polymers, from synthetic or natural origin, or even blends (synthetic-synthetic, synthetic-natural or natural-natural). Nevertheless, polymers are not the only materials used for producing materials in particulate form; across the literature there is a wide array of materials used for the synthesis of particle-based materials, including ceramics and metals. This review deals primarily with polymers and to some extent ceramics. Some examples of polymer-ceramic composites will also be described.

Table I summarizes the most frequently used materials for the synthesis of materials in particulate form. This table also includes the methods for production of these systems and intended applications, with a brief description of the most widely used groups following the table.
Table I. Overview of the materials and methods used for the production of materials in particulate form and envisioned applications. [Information compiled, in the scope of this review, from references 13, 22, 27, 31, 33, 43, 48-21].

<table>
<thead>
<tr>
<th>Material</th>
<th>Type</th>
<th>Method</th>
<th>Application</th>
<th>Description</th>
<th>Ref.</th>
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<td>O/W-solvent evaporation</td>
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<td>Release of Epidermal Growth Factor (EGF)</td>
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<td>Double emulsion technique</td>
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<td>Micro &amp; nanoparticles</td>
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<td>Entrapment of tetanus toxoid for immunization</td>
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<td>Polyethylene glycol (PLA/PEG)</td>
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<td>Microcapsules</td>
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<td>PLGA/Poly-acryloyl hydroxyethyl starch</td>
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<td>PLGA/PCL</td>
<td>Microparticles</td>
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<td>Microspheres</td>
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<td>Polyorthoester (POE)-PLGA</td>
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<td>Water-in-oil-in-water(w/o/w) double emulsion</td>
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<td>Potential for release of water soluble and insoluble drugs</td>
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Dialysis

Polymerization
Suspension polymerization

Nanoparticles

Nanoparticles

Microparticles
Nanoparticles

Microparticles

Poly(methacrylic
acid-g-ethylene
glycol) P( MAA-g-EG)
Poly (trimethylene
carbonate)–poly
(ethylene glycol)–poly
(trimethylene
carbonate) (PTC-PEGPTC)
Polyvinylpyrrolidone
(PVP)
Polyvinyl alcohol
(PVA/P(Vpi/Vac)

Poly(diethylaminoeth

Suspension polymerization

O/W solvent evaporation
Dispersion polymerization
Suspension radical copolymerization
Free-radical solution polymerization

Microparticles

Microparticles

Nanoprecipitation

Nanoparticles

Anionic polymerization in the
presence of series of cyclodextrins
and derivatives
Emulsion polymerization

Nanoparticles

Poly(MePEGcyanoacr
ylate-cohexadecylcyanoacryl
ate)
Polymethyl
methacrylate (PMMA)

Inverse (W/O) emulsion
polymerization
Free radical emulsion polymerization

Microparticles
Nanoparticles
Microparticles

Emulsion polymerization

Diafiltration

Nanoparticles

Nanoparticles

Double emulsion (W-O-W)

Microspheres

Polyisohexylcyanoacr
ylate (PIHCA)

Poly( L-lactic-coglycolic acid) &
Polyethylenoxide
(PLGA-PEO-PLGA)
Poly($ -benzyl Lglutamate)Poly(ethylene oxide)
(PBLG-PEO)
Poly(acrylic acid)
(PAA)
Polyacrylic acid-comethyl methacrylate
Poly(isobutylcyanoacrylat
e)
(PIBCA)

11

Incorporation

Embolic materials

Carrier for antigen

Incorporation &
release

Entrapment & release

Entrapment & release

Incorporation &
release

Adsorption & release
Incorporation &
release
Adsorption & release

Incorporation &
release
Incorporation &
release
Encapsulation

Incorporation &
release

Incorporation &
release

Delivery of the antigen of Aspergillus fumigatus for immune
system response
Introduced through catheters in the management of
gastrointestinal bleeders, traumatic rupture of blood
vessels
Incorporation of glucose-oxidase for treatment of diabetes

Release of methotrexate (anticancer drug)

Release of verapamil
Delivery of HIV-1 Tat protein for vaccination applications
Buformin tosylate — a classical hypoglycemic drug
Release of insulin

Release of tamoxifen

Endocytosis of ampicilin and gentamicin nanoparticles for
intracellular delivery

Ciproflexin (antibiotic)
Release of hematoporphyrin for tumor targeting

Encapsulation of steroid-loaded cyclodextrins

Release of cisplatin

Release of peptides and other hydrophilic drugs

Release of clonazepam (anticonvulsant)

Release of human recombinant erythropoietin

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Chapter I. Materials in Particulate Form for Tissue Engineering


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<th>Physical crosslinking of calcium ions to sodium alginate polymer (gelation) by needle extrusion</th>
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<th>Release of bFGF, glucocorticosteroids, VEGF</th>
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<tr>
<td>Microparticles</td>
<td>Atomization and gelation using Ca\textsuperscript{2+} Microemulsion Gelation using micro-nozzle array Spray-drying Spray-coagulation method</td>
<td>Carrier for cells Purification Incorporation &amp; release</td>
<td>Encapsulation of cells for angiogenic factors release Magnetic affinity absorbents for purification of enzymes Cell encapsulation of human kidney 293 cells Release of L-lactate dehydrogenase enzyme Release of model compounds Incorporation of <em>Aeromonas hydrophila</em> for fish oral vaccination</td>
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<td>Microspherical hydrogels (microspheres)</td>
<td>Gelation using Ca\textsuperscript{2+} Emulsion crosslinking</td>
<td>Carrier for vaccines Incorporation &amp; release</td>
<td>Delivery of several vaccines Incorporation of glucose oxidase for biosensors Delivery of ibuprofen</td>
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Chapter I. Materials in Particulate Form for Tissue Engineering

<table>
<thead>
<tr>
<th>Polyε-caprolactone/poly(methyl methacrylate)</th>
<th>Microparticles</th>
<th>Suspension polymerization</th>
<th>N.A.</th>
<th>N.A.</th>
</tr>
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<tbody>
<tr>
<td>Polyε-caprolactone/poly(ethylene glycol)</td>
<td>Nanoparticles</td>
<td>Polymerization and precipitation</td>
<td>Encapsulation &amp; release</td>
<td>Release of all-trans-retinoic-acid</td>
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<tr>
<td>D-α-tocopheryl polyethylene glycol 1000 succinate/Poly-ε-caprolactone</td>
<td>Microparticles</td>
<td>Double emulsion followed by spray drying</td>
<td>Incorporation &amp; release</td>
<td>Nasal immunization with diphtheria toxoid</td>
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<tr>
<td>Polystyrene</td>
<td>Microparticles</td>
<td>Emulsion solvent evaporation</td>
<td>Incorporation &amp; release</td>
<td>Release of ibuprofen</td>
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</table>

Incorporation & release

Release of superoxide dismutase
Release of nitrofurantoin (antibacterial agent)
Release of Vancomycin
Release of furocortisone acetate for hormonal therapy
Release of diclofenac
Release of nifedipine (calcium antagonist) & propranolol HCl (β-blocker), for treatment of hypertension
Release of 3,4-diaminopyridine (3,4-DAP) for Multiple Sclerosis and Lambert-Eaton myasthenia syndrome
<table>
<thead>
<tr>
<th>Material Type</th>
<th>Carrier Type</th>
<th>Description</th>
<th>Incorporation &amp; Release</th>
<th>Additional Information</th>
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<tbody>
<tr>
<td>Alginate-heparin</td>
<td>Microparticles</td>
<td>N.A.</td>
<td>Incorporation &amp; release</td>
<td>Release of bFGF</td>
</tr>
<tr>
<td>Alginate-poly-L-lysine</td>
<td>Microparticles</td>
<td>N.A. Air atomization Gelation with Ca²⁺ and crosslink</td>
<td>Incorporation &amp; release</td>
<td>Release of NGF Encapsulation of Bifidobacteria for food applications Release of antisense oligonucleotides</td>
</tr>
<tr>
<td>Alginate-poly-L-ornithine</td>
<td>Capsules</td>
<td>Gelation with Ca²⁺ Carrier for cells</td>
<td>Incorporation &amp; release</td>
<td>Simultaneous incorporation of ketoprofen-loaded microspheres and rat pancreatic islets</td>
</tr>
<tr>
<td>Alginate-carboxymethyl chitin</td>
<td>Beads</td>
<td>Dropping the solution into an iron solution</td>
<td>Incorporation &amp; release</td>
<td>Release of model compound (albumin)</td>
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<tr>
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### Chapter I. Materials in Particulate Form for Tissue Engineering

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<td>Microspheres</td>
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<tr>
<td>Starch/Polylactic acid-Bioactive Glass (SPLA/BG 4S55)</td>
<td>Microparticles</td>
<td>Solvent evaporation/extraction</td>
<td>Incorporated &amp; release</td>
<td>Potential for release of bioactive agents &amp; For scaffold materials</td>
</tr>
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N.A. Information not available
The use of synthetic polymers as carriers has predominantly focused on polyhydroxyalkanoates\textsuperscript{221}, in particular poly(\(\alpha\)-hydroxy esters), because the material has long been used in sutures\textsuperscript{222,223}. The most widely used poly(\(\alpha\)-hydroxy esters) polymers for particle-based strategies are polylactide (PLA), polyglycolide (PGA) and their copolymers (poly-DL-lactide-co-glycolide) (PLGA)\textsuperscript{222,224,225}. Their widespread use stems from the ability of these materials to serve a multitude of purposes and applications.

PLA nanoparticles, in general, have the advantage to be able to pass through the capillary bed and to be mainly concentrated in the liver (60-90%), spleen and lungs (2-10%) and to a lower degree, blood marrow\textsuperscript{226,227}. For PLA nanoparticles injected subcutaneously or intramuscularly, they are able to reside at the injection site until biodegradation yields a certain critical molecular weight that enables removal of degradation products\textsuperscript{228}. These particular traits render these systems very interesting for drug delivery applications. Furthermore, tuning of biodegradability can be performed by blending PLA and PGA in a copolymer (PLGA), and by changing the proportion of each of these materials in the copolymer\textsuperscript{229-231}, as PLA degrades much slower than PGA. Degradation of PLA and PLGA is known to proceed by hydrolytic scission of the polymer chain and depolymerization is influenced by molecular weight (Mw), polydispersity and crystallinity\textsuperscript{232,233}.

Although PLGA represents the “gold standard” (exemplified by more than 500 patents) of biodegradable polymers, increased local acidity because of breakdown products of these polymers can lead to irritation at the target site and may also be detrimental to the stability of protein bioactive agents\textsuperscript{230}. Additional potential problems with these synthetic materials include poor clearance – particularly for high molecular weight polymers – and chronic inflammatory response\textsuperscript{232,234}. For this reason, research has been focusing on other synthetic materials, such as Poly(\(\varepsilon\)-caprolactone) (\(\varepsilon\)-PCL), which was, for instance, found to meet the requirements of a biodegradable reservoir or monolithic device for controlled drug delivery, especially in the contraceptive field\textsuperscript{116,235}.

*Polyorthoesters* (POE) have been under development since the 1970s, and they are unique among all biodegradable polymers, as choosing appropriate diols or mixture of diols in their
synthesis can readily vary many of their properties. A number of applications have been found for this class of polymers, such as delivery of 5-fluorouracil, periodontal delivery systems of tetracycline and pH-sensitive polymer systems for insulin delivery\textsuperscript{230,236}. Polyanhydrides have been considered to be useful biomaterials as carriers of bioactive agents to various organs of the human body, such as bone tissue, blood vessels, brain and eyes\textsuperscript{237}. They can be prepared easily from available, low cost resources, can be manipulated to meet desirable characteristics, are biocompatible and degrade \textit{in vivo} into non-toxic diacid counterparts that are eliminated from the body as metabolites\textsuperscript{237}. But synthetic materials do not completely fulfill the needs in terms of biomedical applications, and in recent years, many researchers have been turning their research focus to natural origin materials, as these might obviate several of the drawbacks of synthetic ones. Polyaminoacids [such as poly(\(\gamma\)-methyl-L-glutamate)] that have already shown good biocompatibility, have been investigated for the delivery of low molecular-weight compounds\textsuperscript{230,238}. Their widespread use however, is limited by their antigenic potentials and some difficulties in the control of release that might arise from the dependence on enzymes for biodegradation. Collagen, namely type I collagen, is the most widely used natural polymer, and typically derived from bovine or porcine bone, skin or tendon\textsuperscript{239}. The fact that collagen is from animal origin raises concerns, such as the possibility of transmitting diseases. This is particularly critical for materials from bovine sources, due to malignancies such as Bovine Spongiform Encephalopathy (BSE) and the human variant, the Creutzfeldt-Jakob disease (CJD). For this reason, other sources of collagen, namely recombinant, are seen as an alternative. Collagen exhibits biodegradability, weak antigenicity and superior biocompatibility\textsuperscript{24,240}. This material is regarded as very promising for the delivery of growth factors, as it was found that an electrostatic interaction was the main driving force for the complexation between acidic gelatin and basic fibroblast growth factor (bFGF)\textsuperscript{24}. Biodegradable collagen based nanoparticles or nanospheres are thermally stable and readily sterilizable\textsuperscript{24,241}. Moreover, nanoparticles can be taken up by the reticuloendothelial system\textsuperscript{242}, and enable an enhanced uptake of exogenous compounds, such as anti-HIV biologically active agents, by a number of cells, especially macrophages\textsuperscript{243}, which may be an
additional advantage of collagen based nanoparticles as a systemic delivery carrier\textsuperscript{24}. Coupled to a small size and a large surface area, high adsorptive capacity and ability to disperse in water to form a clear colloidal solution, the potential of collagen-based nanoparticles has been demonstrated in their use as a sustained release formulation for anti-microbial agents or steroids\textsuperscript{24}. However, some disadvantages of collagen-based systems comprehend the difficulty of assuring adequate supplies, poor mechanical strength\textsuperscript{244}, problems related to the use of animal origin, in particular bovine collagen, due to the possibility of disease transmission. Alternatives to animal origin collagens – those produced by recombinant technologies – still present a high cost.

*Hyaluronan* (hyaluronic acid), typically derived from rooster combs, is a minor component of bone extracellular matrix (ECM)\textsuperscript{232}. It has been used as a carrier for Bone Morphogenetic Proteins (BMPs) and sodium hyaluronate gel was used as the delivery system for bFGF\textsuperscript{232}. One advantage of hyaluronic acid is that it is negatively charged and can form ionic bonds with positively charged BMPs to increase affinity. Disadvantages of hyaluronic acid include rapid resorption unless crosslinked or chemically modified to decrease its intrinsic hydrophilicity\textsuperscript{232}. However, the fear that some of these materials might additionally be carriers for diseases has lead researchers to find other sources of natural origin products, mostly from plant origin and produced by microorganisms. These might present additional advantages such as supply, low cost, ability to be processed by several methodologies and to tailor their properties.

In this field of polymers from nature, poly(glucoses) like *starch* and *dextran* have long been used for encapsulating materials for pharmaceutical, cosmetic or food applications\textsuperscript{245-248}. *Dextran* are being actively investigated for sustained delivery of therapeutic and imaging agents, particularly for injectables and colon-specific DDSs. *Starch*-based polymers have been proposed by Reis *et al.*\textsuperscript{249} as materials with potential for biomedical applications, particularly as scaffolds for bone tissue engineering applications\textsuperscript{250,251}, bone cements\textsuperscript{252,253} and recently as drug delivery systems\textsuperscript{193,254}. These materials have been shown to be biocompatible *in vitro*\textsuperscript{255,256}, and to possess a good *in vivo* performance\textsuperscript{257,258}. A very important feature of most natural origin materials, besides the ones described above, is the reaction of the host to degradation products (in the case of starch, degradation products are oligosaccharides that can be readily metabolized to produce
energy). Regarding their biodegradability, enzymes typically catalyze the hydrolysis of natural biodegradable polymers, for instance, \(\alpha\)-amylase catalyzes the hydrolysis of starch, which may constitute a strategy to tailor the biodegradability of the material\(^{189,259,260}\).

**Chitosans** are promising natural polymers that show biocompatibility, good absorption-enhancing, controlled release\(^{14,157,230}\), bioadhesive properties\(^{230}\), as well as cell culture, enzymatic immobilization and chromatograph support\(^{29}\). Chitosan is a product from deacetylation of chitin, produced with varied degrees of deacetylation, and its utilization is only limited by the poor/in-solubility of chitosan in water\(^{261}\). However, growing attention given to this material for several applications, not only drug delivery, makes us believe that chitosan holds promise to become a very successful material for biomedical applications.

Another widely used polymer of natural origin is **alginate**, a natural polysaccharide extracted from brown algae and composed of various proportions of \(\beta\)-D-mannuronic acid (M) and \(\alpha\)-L-guluronic acid (G) residues. This naturally occurring biopolymer has many applications in various areas of biosciences and biotechnology (e.g. as a matrix for the entrapment and/or delivery of a variety of proteins and cells), and in the food and beverage industry (as a thickening or a gelling agent and a colloidal stabilizer)\(^{130,132,262}\). Besides the most known method to prepare alginate beads - which is a gelation method were a sodium alginate solution is single dropped into a calcium solution, forming particles with several \(\mu\)m in diameter - several other well-known methods (atomization, spraying and water-in-oil emulsification methods) can also be used to prepare alginate microparticles that are less than 200 \(\mu\)m in diameter\(^{132,263}\). Gelation occurs by an ionic interaction between the calcium ions and the carboxylate anions of G – G blocks as calcium ions diffuse from the external source into the droplet\(^{130}\). The main advantage of using alginate is that the alginate gelation process occurs under very mild conditions without using high temperatures or chemical crosslinking agents\(^{130}\), thus allowing the preservation of the viability and biological activity of the entrapped cells and other agents, respectively. However, the application of this system has been limited by the poor mechanical stability. Combining alginate with other polymers and ceramic materials has been shown to obviate this feature\(^{217}\). Recent studies
described a dual function of alginate microparticles, both as carriers for cells and drugs, for application in diabetes\textsuperscript{144}, an idea that we also propose for bone tissue engineering applications using starch-based microparticles\textsuperscript{264,265}.

Polyhydroxybutyrate is a polyester produced as granules by microorganisms\textsuperscript{266-268} and has been widely studied for tissue engineering applications\textsuperscript{269}, mainly for scaffold materials in combination with ceramic materials\textsuperscript{270-274} and also as a vehicle for drug delivery\textsuperscript{275,276}.

Although polymers are seen as the most versatile class of materials, other classes of materials have been widely studied for biomedical applications. Among these are ceramic materials, which are refractory, polycrystalline compounds, composed of ionically bonded compounds\textsuperscript{277,278}. Ceramic materials, such as \textit{tricalcium phosphate} (TCP), \textit{hydroxyapatite} (HA) and \textit{bioactive glasses} (BG) have been widely investigated for hard tissue applications\textsuperscript{204-208,279-286}, for filling, support and promotion of regeneration. Their role as drug delivery devices derives from their compatibility and physical characteristics, such as non-immunogenic and degradability. Ceramics as drug delivery systems were basically in the form of porous materials and using the above-mentioned well-known ceramics. As proposed by Ducheyne and co-workers\textsuperscript{287-289}, sol-gel technology for the formation of silica-based xerogels, which allows the introduction of functional proteins into glass-like materials, is a very interesting strategy, that couples a bioactive behavior from these systems to drug delivery capability and ability of additionally tailoring other properties. Another major advantages relate to room temperature processing without the need for solvents.

Further details on ceramic materials in bone tissue engineering can be found on the second part of this review\textsuperscript{290}.

5. APPLICATIONS

Although some applications of materials in particulate form have been mentioned so far, table II lists the major applications of materials in particulate form in the biomedical field.
Table II. Major applications of materials in particulate form in the biomedical field. [Information compiled, in the scope of this review, from references 32, 42, 50-55, 58-66, 100, 107, 111, 129, 130, 150-154, 175, 193, 201-206, 209, 213, 214, 210, 283-305].

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<td>Drug delivery</td>
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</table>

By far the greatest field of application for materials in particulate form, as found in the literature, is as drug delivery systems (DDS) and a few important principles regarding this field follow.

5.1. Basic Concepts on Drug Delivery

Drug delivery routes are normally four\textsuperscript{15,314}: (i) oral, for pills and syrups (ii) rectal, (iii) intramuscular or intravenous, for solutions and (iv) topic, as for eye drops. These conventional systems of drug delivery have a major disadvantage, being that along time the concentration of bioactive agent decreases to a minimum, leading to the need of a new dose of bioactive agent in a short time interval; another problem is that the bioactive agent will be distributed systemically, throughout the body of the patient\textsuperscript{15,315}. In general, for oral drug delivery systems, the major problem is the quick loss of activity of the therapeutic agent in the hostile environment of the stomach\textsuperscript{16,316,317}. It was also observed that chemically attaching a bioactive agent to a polymer (bioactive agent/macromolecule conjugate) may alter such properties as its distribution in the body, rate of appearance in certain tissues, solubility or antigenicity\textsuperscript{15,29}. 

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Since oral drug administration remains the easiest and the most comfortable method\textsuperscript{7,316-318}, the microencapsulation of bioactive agents seemed to be an alternative to overcome the problem, allowing their slow release and protection against the acidic and enzymatic gastric environment\textsuperscript{32,316}. All these were reasons that led to the development of delivery systems, whose aim is to facilitate the dosage and duration of effect of the bioactive agent, causing minimal harm and improving patient compliance\textsuperscript{7,15}, since they would allow the reduction of the dosage frequency\textsuperscript{29,230}.

For drug delivery applications, the development of intravenously administrated carriers with blood circulation times long enough to continuously deliver bioactive compounds\textsuperscript{20,29,36,40} imaging agents or other entities to specific sites of action\textsuperscript{40} has been a major challenge, since these carriers must possess a set of features compatible with the task to perform. The desired features of such a carrier include\textsuperscript{22,40} 1) that the agent to be encapsulated comprises a reasonably high weight fraction (loading) of the total carrier system (for example more than 30%), 2) the amount of agent used in the first step of the encapsulation process is incorporated into the final carrier (entrapment efficiency) at a reasonably high level (for example, more than 80%), 3) the ability to be freeze-dried and reconstituted in solution without aggregation, 4) biodegradability, 5) small size and 6) characteristics to prevent rapid clearance of the particles from the bloodstream.

Also, within drug delivery systems, it is essential to distinguish between sustained and controlled delivery systems, as these two types denote very different applications. Sustained systems imply that the bioactive agent is delivered over a prolonged period of time to overcome the highly periodic nature of tissue levels associated with conventional (enteral or parenteral) administration of single doses by tablets or fluids\textsuperscript{15,315,319}. The term "controlled" is used generically to indicate any device in which some control is exerted over the way in which the bioactive agent is delivered to the tissues once it has been administrated to the patient\textsuperscript{15,315,319}. This is best exemplified in the concept of thermally and pH-responsive materials, where variation in the temperature/pH changes discontinuously or sharply properties such as volume\textsuperscript{19,111,320}. This concept is extremely important, as it can be used as a means to trigger the release of the entrapped bioactive agent, and thus allow one to exert control over the system.
If other ways of controlling the system can be developed, besides temperature and pH, for instance the presence of a certain agent would trigger the release of the incorporated agent, this could be used for other applications. One such application is described by Cavanaugh et al.\textsuperscript{321}, where the microparticles released their load of adenovirus only upon cell contact, thus preventing inactivation of the viral load.

### 5.2. Polymers as the primary choice for DDS

The class of materials that has been most widely studied for drug delivery applications is the polymeric one. Polymeric delivery systems generally release bioactive agents by the following mechanisms\textsuperscript{15,316}: diffusion, chemical reaction or solvent activation. The release of a bioactive agent from a matrix is primarily controlled by diffusion of the bioactive agent through the polymer, being erosion of the polymer an additional but important factor\textsuperscript{16}. For biodegradable polymers, degradation is a chemical process, whereas erosion is a physical phenomenon dependent on dissolution and diffusion process. As soon as the bioactive agent-containing polymer (A) comes into contact with the external liquid environment, it enters the polymer matrix (B), resulting in a swelling process (C), which allows the diffusion of the bioactive agent into the external environment\textsuperscript{16} (D), as illustrated in figure 1. Factors influencing the release rate include the molecular size of the bioactive agent and loading percentage into the polymer, as well as polymer composition, molecular weight and the dimensions and shape of the matrix\textsuperscript{15}.

![Figure 1. Schematics of the release of entrapped bioactive agents from biodegradable polymeric particles. When the polymer device incorporating the active agent (A) is inserted into the](image-url)
environment, the fluid from the surrounding medium enters the matrix (B), causing swelling of the device (C). The fluid creates diffusion channels (C) and the incorporated active agent is released to the external environment (D). In the case of biodegradable polymers, the device removal will occur by the degradation of the material.

There are usually three distinct phases of release for biodegradable polymers, as shown in figure 2: 1) a burst or initial period of rapid diffusion of active agent located close to the surface of the polymer, 2) a period of minimal release, during which the polymer is gradually hydrolyzed in bulk but has not yet decreased sufficiently in molecular weight (Mw) to allow an increased diffusional release of the active agent and 3) the Mw of the polymer is sufficiently low to allow its solubilization in the aqueous environment, and the release of the remaining active agent occurs as the polymer is eroded.

Figure 2. Release profile for biodegradable polymers. The first stage (1) is a burst release, caused by diffusion of the bioactive agent located closer to the surface. The second stage (2) is caused by gradual degradation of the polymer and the third stage (3) is characterized by massive degradation (solubilization) of the material.

This release profile is generally regarded as a problem common to many biodegradable systems, where the release is dependent upon degradation of the system with time, thus not being able to achieve any type of control. This type of device is therefore more adequate for sustained rather than controlled release.
In short, and for drug delivery systems in general, bulk properties of the polymer that need to be considered include\textsuperscript{15,230}:

- Molecular weight
- Physical properties (bioadhesiveness, mechanical stability)
- Solubility based on the release mechanism (diffusion or dissolution-controlled) and
- Site of action.

Bioadhesiveness need to be taken into account when drug delivery systems are targeted to mucosal tissues, whereas polymers for ocular devices have to be aqueous or lipid-soluble in addition to having good film-forming ability and mechanical stability for good retention. Structural properties of the matrix, its micromorphology and pore size are important with respect to mass transport (of water) into and (of bioactive agent) out of the polymer\textsuperscript{230}.

Of great importance is, however, the assurance that the biological activity of the incorporated agent is preserved throughout manufacturing, storage, delivery and release\textsuperscript{323}. This, together with the release profile, is of particular importance when designing a delivery system, because as much as the release profile is adequate, there is no point of having it if the biological activity of the agent to be delivered is lost during processing. This idea is mostly coupled with the use of solvents in the production of the delivery system because, as mentioned before, organic solvents might cause inactivation of the agent to be loaded into the system. For growth factors, BSA has been shown to be protective when used as an adjuvant during the loading process\textsuperscript{98,324}, but there is the necessity of methods that obviate this step.

Regarding the release profile, strategies to control or render it more adequate for a particular application, by means of modifying parameters such as the surface (by coating, chemical modification) or creating dual-release systems (layers of materials that can incorporate different molecules)\textsuperscript{324,325} can greatly improve the properties of several materials, and should be actively pursued.
6. CONCLUSIONS

Materials in the particulate form have been employed into a diversity of biomedical applications. This derives from their properties, such as size, surface area, and physicochemical properties, which stem from the diverse materials and methods combined for their production. Within the range of applications, drug delivery has had a highlighted role, because it poses itself as a means of overcoming limitations inherent to conventional delivery methods. Currently, the use of these systems in innovative strategies, where they can play a multitude of roles - delivery of bioactive agents, structural support and carriers of cells - makes it mandatory for researchers to become even more creative to develop such a system. Within this perspective, an area of tissue engineering that can obviously benefit from the specific properties of materials in particulate form is bone tissue engineering.

Part B of this review deals with the roles – played and potential - of particle-based systems in this specific subset of tissue engineering applications – bone tissue engineering.
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Chapter I. Materials in Particulate Form for Tissue Engineering


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Chapter I. Materials in Particulate Form for Tissue Engineering


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Abstract

Materials in particulate form have been the subjects of intensive research in view of their use as drug delivery systems. While within this application there are still issues to be addressed, these systems are now being regarded as having a great potential for tissue engineering applications.

Bone repair is a very demanding task due to the specific characteristics of skeletal tissues, and the design of scaffolds for bone tissue engineering presents several difficulties. Materials in particulate form are now seen as a means of achieving higher control over parameters such as porosity, pore size, surface area and mechanical properties of the scaffold. These materials also have the potential to incorporate biologically active molecules for release and to serve as carriers for cells. It is believed that the combination of these features would create a more efficient approach towards regeneration.

This review focuses on the application of materials in particulate form for bone tissue engineering. A brief overview of bone biology and the healing process is also provided in order to put the application in its broader context. An original compilation of molecules with a documented role in bone tissue biology is listed, as they have the potential to be used in bone tissue engineering strategies. To sum up this review, examples of works addressing the above aspects are presented.

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*This part is based on the following publication:
1. Materials in particulate form and bone tissue engineering

Regarding materials for use in bone Tissue Engineering (TE), several approaches have been shown to be effective in stimulating bone regeneration, and ceramics specially excel in this regard. Notwithstanding the stimulatory effect of bioactive ceramics on bone tissue formation, there is a continuous need to explore avenues wherein materials, cells and biologically active molecules are combined. This is critical, since cells and growth factors are two key elements when discussing bone biology/healing, being their interaction fundamental for an effective regeneration process. Although continuous progress is being made in understanding osseous healing process, these new insights have not readily found their way into effective tissue engineering approaches. The combination of materials, cells and growth factors seems to be the recipe for a truly effective bone TE strategy. Therefore, the present review focuses on the role that particle-based systems can play in bone TE, emphasizing the combination of materials with cells and their role as carriers for biologically active molecules.

2. Requirements for an effective bone TE strategy

The skeletal system has been described as a dynamic, mineralized vascular tree that serves as a metabolic reservoir of calcium as well as a structural scaffold for neurovascular distribution and muscular function. Important properties that are part of the skeletal system are:

- It is the reservoir of calcium in the body, containing 99% of the body's calcium;
- Its homeostasis is regulated to a large degree by systemic influence expressed through the endocrinal system, but also controlled at the local level;
- Its structural function derives from its nature as mineralized tissue;
- It is an anisotropic material (the mechanical properties vary according to the direction);
- Its physiological efficiency is evidenced by the maximum strength with minimum mass;
- It has a relative high turnover (remodeling) rate in young individuals.
Chapter I. Materials in Particulate Form for Tissue Engineering

The ultimate goal of bone tissue engineering is to recapitulate the structure and function of the native tissue it is designed to replace. Therefore, the following principles apply to scaffolds for bone tissue engineering:

- **Bone TE scaffolds require not only a material with adequate composition, but also mechanical stability, precise shapes and tailored pore distribution**.

The osseous tissue is an exquisitely structured composite material: it is composed of organic and inorganic components, and it also contains water. The inorganic component is apatitic calcium phosphate, which comprises 60-70% of the bone dry weight. The organic component contains materials such as collagen, extracellular matrix proteins (osteocalcin, osteonectin, bone sialoprotein), tissue specific cells and water. Having this in mind is crucial for the design and fabrication of an adequate scaffold. The adult skeleton consists of cortical (or compact) and trabecular (or cancellous, spongy) bone, which are present in various ratios and geometries to form the individual bones of the body. Both cortical and trabecular bone tissue types are essential for the ability of skeleton to provide structural support that can simultaneously withstand torsion and bending. A minimum pore size is required for tissue growth, interconnectivity for access to nutrients and transport of waste products, pore shape and roughness for better cell spreading and pore throat size for passage of tissue throughout the scaffold. The lack of adequate porosity can lead to failure, as inner areas of the scaffold will lack adequate nutrient and oxemic conditions to allow cells to populate those areas.

- **The material should act as a permissive environment into which bone cells would be enticed to migrate and begin the process of depositing bone matrix in the carrier template**.

Bone, being a mineralized tissue that is incapable of internal expansion or contraction, can only be remodeled along the surface via anabolic and catabolic modeling. Bone is resorbed by osteoclasts and formed by osteoblasts, and the coupling of these two processes underlies bone remodeling. Figure 1 depicts the bone healing process, which the repair using scaffold materials
attempts to mimic. Briefly, upon fracture and formation of a blood clot, the fibroblast layer of the periosteum begins a period of active division in order to generate enough cells to close the gap at the surface. In the central zone of the bone, haematopoietic precursors in the bone marrow differentiate into osteoclasts that start the process of resorbing the end bone of the defect, and mesenchymal cells within the bone marrow are stimulated to migrate to the healing site. These cells originate chondrogenic cells that produce an intermediate cartilaginous matrix that mineralizes. This cartilaginous phase is then replaced by new bone synthesized by osteoblasts. This newly formed bone is the so-called woven bone, which possesses an unorganized structure and still need to be remodeled by the normal osteoclast-osteoblast process. [This scheme does not incorporate the vascularization process.]

To be successful, a scaffold material must be capable of allowing a similar process to occur. Ideally, the scaffold would degrade at a similar rate at which the tissue is healing, and the new tissue would fully replace the space once occupied by the scaffold.
Chapter I. Materials in Particulate Form for Tissue Engineering

Hematopoietic cell

Mesenchymal stem cell / Bone marrow stromal cell

Osteoclast

Chondroblast

Osteoblast
Figure 3. Healing process of bone, depicted in a simplified diagram. After the defect and formation of a blood clot, haematopoietic precursors (H) in the bone marrow differentiate into osteoclasts (OC) that start the process of resorbing the end bone of the defect. Mesenchymal cells (MSCs) within the bone marrow are stimulated to migrate to the healing site. These cells originate chondrogenic cells (CB) that produce an intermediate cartilaginous matrix that progressively mineralizes. This cartilaginous phase is then replaced by new bone synthesised by osteoblasts (OB). Not depicted is the role of vascularization [based on refs 21,22].

- A system designed for bone repair would ideally combine osteoconductive and osteoinductive properties, in a way that new bone formation can be enhanced through an adequately shaped 3-D-scaffold (osteocconduct) and by a biological stimulus (osteoinduction)23.

Ceramic materials, due to their inorganic nature and ionic composition are adequate for bone applications. Examples of ceramic materials are calcium phosphates, such as hydroxylapatite, tricalcium phosphate and bioactive glasses, known for their ability to bond to and stimulate bone regeneration1,24-27. From these, bioactive glass has been shown to stimulate osteogenesis28,29 via surface-mediated and solution-mediated mechanisms30.

Other materials than bioactive glasses have been extensively used, such as β-tricalcium phosphate (TCP)31 and hydroxylapatite32,33, but there are also some reports on the use of composite materials (ceramic-polymer)34. Composite ceramic-polymer materials have the advantages of combining bioactivity, ability of adequate control of the scaffold degradation rate, and enhancement of the mechanical properties and structural integrity of scaffolds35.

- Some biologically active molecules act locally and therefore must be delivered directly to the site of regeneration via a carrier matrix19.

The system should be able to provide not only structural support but also to serve as carrier for biologically active agents that can enhance the regenerating potential of the system. These agents can be of different nature, as listed in table III. Since the identification Bone Morphogenetic
Proteins (BMPs) by Urist\(^{36}\), several other growth factors, as well as hormones and other biologically active agents, have been identified as acting in Bone, and recently been of interest for bone tissue engineering strategies.

**Table III.** Some molecules and trace elements with a brief description of their role/effect on bone. [Information compiled, in the scope of this review, from references 4,8,13,14,22,36-115].

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Role/effect on bone tissue</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone Morphogenetic Proteins</strong> (<strong>BMPs</strong>)</td>
<td>Expressed in bone generation, regeneration, modeling and remodeling.</td>
<td>36-44</td>
</tr>
<tr>
<td>BMP-2, BMP-4, BMP-3, BMP-5, BMP-6, BMP-7(OP-1)</td>
<td>Stimulation of osteoblasts and bone formation.</td>
<td>45, 46, 47</td>
</tr>
<tr>
<td><strong>Epidermal Growth Factor</strong> (<strong>EGF</strong>)</td>
<td>Stimulates chondrocyte proliferation while decreasing the ability of cells to synthesize matrix components</td>
<td>45, 46, 47</td>
</tr>
<tr>
<td><strong>Basic Fibroblast Growth Factor</strong> (<strong>bFGF</strong>)</td>
<td>Mitogenic effects on cells from the mesenchymal lineage. Promotes proliferation and inhibits differentiation. Involved in fracture repair</td>
<td>13, 45-47</td>
</tr>
<tr>
<td><strong>Insulin-like Growth Factor</strong> (<strong>IGF</strong>)</td>
<td>Enhances osteoblast activity and chemotaxis. Type I collagen production, decreases collagen degradation, stimulates cell growth in various cell types and blocks apoptosis. Induction of bone formation. Enhancement of VEGF expression in osteoblasts.</td>
<td>13, 48-49</td>
</tr>
<tr>
<td><strong>Platelet Derived Growth Factor</strong> (<strong>PDGF</strong>)</td>
<td>Potent mitogen and chemotaxic factor for cells of mesenchymal origin. Anabolic action on bone formation <em>in vivo</em></td>
<td>50-52</td>
</tr>
<tr>
<td><strong>Transforming Growth Factors-β</strong> (<strong>TGFs-β</strong>)</td>
<td>Mitogenic and chemotactic effects; increase in collagen and extracellular matrix synthesis. New bone formation. Involved in fracture repair. May promote osteoclast apoptosis. Overexpression leads to osteoclast mediated resorption. Potent inhibitors of terminal differentiation of epiphyseal plate chondrocytes.</td>
<td>13, 46, 51, 53-56</td>
</tr>
<tr>
<td><strong>Hepatocyte Growth Factor</strong> (<strong>HGF</strong>)</td>
<td>Contributes to fracture repair by upregulating the expression of BMP receptors</td>
<td>63</td>
</tr>
<tr>
<td><strong>Vascular Endothelial Growth Factor</strong> (<strong>VEGF</strong>)</td>
<td>Induces vascularization</td>
<td>64-67</td>
</tr>
<tr>
<td><strong>Calcitonin</strong></td>
<td>Secreted by the thyroid gland controls the levels of calcium and phosphorous in the blood. When administrated inhibits bone resorption by decreasing the number of osteoclasts and their resorptive activities. Effectively inhibits the manifestations of metabolic bone disorders such as Paget's disease and osteoporosis by frequent and relatively high dosage.</td>
<td>68-71</td>
</tr>
<tr>
<td><strong>Melatonin</strong></td>
<td>Increased proliferation of osteoblastic cells and increased procollagen type I c-peptide production. Augmented gene expression of sialoprotein and other bone marker proteins like alkaline phosphatase and osteocalcin in bone cells. Modifies bone remodeling after ovariectomy in close relation with estradiol.</td>
<td>72-73</td>
</tr>
<tr>
<td><strong>Parathyroid hormone</strong> (<strong>PTH</strong>)</td>
<td>In low dose causes increase in bone density and cancellous/trabecular bone volume without impairing normal bone architecture and has a direct effect on osteoblasts’ recruitment/proliferation</td>
<td>68-74, 79</td>
</tr>
<tr>
<td><strong>Thyronin</strong></td>
<td>Thyroid hormone which stimulates osteoclastic bone resorption</td>
<td>14</td>
</tr>
<tr>
<td><strong>Cortisol</strong></td>
<td>Influences PTH-responsiveness of bone. Inhibitor of the stimulatory effect of IGF-1</td>
<td>80-82</td>
</tr>
<tr>
<td><strong>Interleukin-6 (IL-6)</strong></td>
<td>Stimulates the differentiation of osteoclasts from hematopoietic precursors</td>
<td>83-84</td>
</tr>
<tr>
<td><strong>Interleukin-1 (IL-1)</strong></td>
<td>Stimulates the effect of IL-6. Most potent inducer of bone resorption</td>
<td>8, 85-87</td>
</tr>
<tr>
<td><strong>Tumor Necrosis Factor</strong> (<strong>TNF</strong>)</td>
<td>Stimulates the effect of IL-6. Stimulates bone resorption and suppresses its formation</td>
<td>88-91</td>
</tr>
<tr>
<td><strong>Prostaglandin E2</strong> (<strong>pE2</strong>)</td>
<td>Potentates the effect of IGF-1. Concentration dependent actions</td>
<td>92-95</td>
</tr>
</tbody>
</table>
Two groups of molecules (growth factors and steroids) with well documented effects over bone, and considered relevant to the field of bone tissue engineering, are described below.

<table>
<thead>
<tr>
<th>Interferon-β (IFN-β)</th>
<th>Suppresses osteoclastogenesis and bone resorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon-γ (IFN-γ)</td>
<td>Suppresses bone resorption induced by IL-1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biphosphonates</th>
<th>Considered stable analogs of pyrophosphate, a physiological regulator of calcification and bone resorption. Decrease bone resorption/increase bone mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etidronate Clodronate Pamidronate Alendronate Ibandronate Risedronate Zoledronate Tiludronate YH 529 Icadronate Olpadronate Neridronate EB-1053</td>
<td>Decreases the level of tumor necrosis factor alpha (TNFα-) in the bone marrow of rats with adjuvant arthritis</td>
</tr>
<tr>
<td>TRK-300</td>
<td>Synthetic flavonoid derivative that improves osteoblast cell activity inhibiting bone resorption</td>
</tr>
<tr>
<td>Ipriflavone (Isoflavone)</td>
<td>Anti-inflammatory and anti-osteoclastic activity</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>Regulates osteoblast differentiation by either activating or repressing transcription of numerous bone phenotypic genes</td>
</tr>
<tr>
<td>Vitamin D and analogues</td>
<td>Regulates osteoblast differentiation by either activating or repressing transcription of numerous bone phenotypic genes</td>
</tr>
<tr>
<td>TAK-778 [ (2R,4S)-(−)-N-(4-diethoxyphosphoryl)methyl-phenyl]-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide]</td>
<td>TAK-778, a benzothiepin derivative, increased cellular alkaline phosphatase activity, an index of bone formation, in a culture of rat bone marrow stromal cells, and enhanced the action of BMP in mouse osteoblastic cell line MC3T3-E1</td>
</tr>
<tr>
<td>TP508 (thrombin peptide)</td>
<td>Activates angiogenesis-related genes during femoral fracture healing. Regulates BMP-2 and-7 expression by human osteoblasts. Enhances bone formation</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Found to inhibit osteoclasts and to decrease the resorptive area</td>
</tr>
<tr>
<td>Corticosteroids (Glucocorticoids)</td>
<td>Excess generally associated with net bone loss, due to decrease in bone formation and increase in bone resorption</td>
</tr>
<tr>
<td>Statins</td>
<td>Generally used for inhibiting HMG CoA reductase (rate limiting step in cholesterol synthesis), Enhance transcription of BMP-2 in bone cells</td>
</tr>
<tr>
<td>Estrogen/Testosterone</td>
<td>Deficiency results in high turnover of bone remodeling in which the accelerated bone resorption and formation simultaneously occur, but with resorption exceeding formation. Protective effect on bone tissue mass</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Trace elements</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoride</td>
<td>Anabolic effects on bone, but has a narrow toxic-therapeutic window</td>
</tr>
<tr>
<td>Strontium</td>
<td>Potential increase in bone mass</td>
</tr>
<tr>
<td>Aluminum</td>
<td>Causes mineralization deficit by inhibiting hydroxyapatite crystal formation. Interferes locally with osteoblast maturation</td>
</tr>
<tr>
<td>Boron</td>
<td>Deficiency causes osteopenia. Intervene in magnesium metabolism. Interact with calcium and other ions.</td>
</tr>
<tr>
<td>Tin</td>
<td>Significant for coupling-uncoupling of the remodeling process</td>
</tr>
<tr>
<td>Zinc</td>
<td></td>
</tr>
</tbody>
</table>

Chapter I. Materials in Particulate Form for Tissue Engineering
Growth factors

Among all available growth factors PDGF, IGF, VEGF, TGF-β and BMPs appear to have the closest association with bone regeneration. PDGF plays an important role in inducing proliferation of undifferentiated cells in mesenchymal tissues. It can enhance bone regeneration in conjunction with other growth factors namely IGF, TGF-β or BMP, but is unlikely to provide entirely osteogenic properties itself. IGFs have an important role in general growth and maintenance of the body skeleton, and appear to integrate and extend the effects of both BMPs and TGF-βs. Equally important is VEGF, which couples ossification and angiogenesis during bone formation. BMPs are thought to have their major effects on early precursor bone cell replication and osteoblast commitment. In contrast, TGF-βs are thought to be the most potent inducers of committed bone cell replication and osteoblast matrix production.

(i) Bone Morphogenetic Proteins

Growing interest on the clinical use of BMPs as means of promoting bone formation has lead to extensive studies on this group of growth factors. In brief, BMPs are hydrophobic, low molecular weight, dimeric molecules with two polypeptide chains held together by a single disulfide bond. The name stems from the demonstration of a hydrophobic noncollagenous glycoprotein that induced mesenchymal-type cells to differentiate into a spherical ossicle with a medulla containing hematopoietic bone marrow. This family of secreted growth factors forms a subgroup of molecules within the transforming growth factor β (TGF-β) superfamily. The history of BMP evolved from observations of allogenic bone matrix-induces cartilage and bone development in mammalian species. In embryogenesis, BMPs appear to be omnipresent, being observed in nearly all developing visceral and somatic organs. At least two distinct pathways mediate BMP signaling: the Smad pathway and the mitogen-activated protein kinase (MAPK) pathway.
(ii) Platelet-Derived Growth Factor

Effects by platelet-derived growth factors (PDGFs) are generally limited to situations associated with inflammation and repair. However, PDGF has been shown to be involved in the chemotaxis of osteoblast precursors to the site of bone regeneration. In vitro, it has been shown to stimulate migration and to increase the proliferation rate of osteoblasts, reducing alkaline phosphatase activity and inhibiting bone matrix formation.

There are three isoforms, characterized by the combination of A- and B-chains featuring two homodimeric (PDGF-AA and PDGF-BB) and one heterodimeric isoform (PDGF-AB). PDGF-BB and PDGF-AB are systemically circulating isoforms contained in alpha granules of platelets from where they are released after adhesion of platelets to injured sites of vessel walls, whereas PDGF-AA is secreted by unstimulated cells of the osteoblastic lineage.

The biochemical effects of the different isoforms appear to be graded according to their binding characteristics to the surface receptors. In osteoblast-enriched environments, receptors that favor binding of PDGF-BB chains preferably mediate these effects. PDGF may thereby contribute to recruitment of bone cells during remodeling and repair, as it is deposited in bone matrix from where it is released during matrix degradation.

The effectiveness of PDGFs on osteoblasts is rapidly modulated by inflammatory cytokines, causing changes in specific PDGF receptors. The activated receptors lead to the activation of the MAPK cascade, resulting in the transcription of important genes related with bone formation.

Corticosteroids

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex. They are involved in a wide range of physiologic systems such as stress response, immune response
and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behavior. This class of molecules is often used as part of the treatment for a number of different diseases, such as severe allergies or skin problems, asthma, or arthritis. Within corticosteroids there are mineralocorticoids and glucocorticoids, and a brief description on the latter follows.

(i) Glucocorticoids

Glucocorticoids such as cortisol control carbohydrate, fat and protein metabolism and are anti-inflammatory by preventing phospholipid release, decreasing eosinophil action and a number of other mechanisms.

Physiological amounts of glucocorticoid tend to have permissive effects on osteoblasts\textsuperscript{45}. However, either when endogenously in excess or when administered exogenously, glucocorticoids lead to a dramatic decrease in bone mineral density. Whereas chronic glucocorticoid exposure suppresses bone formation and disrupts resorption and the bone remodeling cycle, major detrimental effects on the skeleton occur from a decrease in osteoblast replication, bone matrix protein synthesis, marked decrease in osteoblast gene transcription and skeletal tissue loss\textsuperscript{61,130}. Pharmacological doses of the glucocorticoids cortisol and dexamethasone directly lower basal IGF-I expression\textsuperscript{131}, and in vitro studies revealed that high excess glucocorticoid suppresses the expression of IGF-I and the type TGF-b receptor (TGF-3RI) by osteoblasts, consistent with decreases in specific aspects of osteoblast function\textsuperscript{61}.

Dexamethasone is a synthetic member of the glucocorticoid class of hormones. It acts as an anti-inflammatory and immunosuppressant, with potency about 40 times that of hydrocortisone\textsuperscript{132-134}. In vitro, dexamethasone has been employed as a differentiation agent for bone marrow cells to progress into the osteoblastic lineage\textsuperscript{135}. Within this last role, strategies employing the incorporation of dexamethasone in polymeric materials to be used as carriers for the differentiation of cells into the osteoblastic lineage have been described in the literature\textsuperscript{136}, which confers
dexamethasone an highlighted role in bone TE approaches.

3. Materials in particulate form: towards bone TE

In recent years there has been interest on the fabrication of 3-D systems using a microsphere-based approach for a TE scaffold possessing a porous interconnected structure, with the incorporation of ceramics to control the mechanical properties of the sintered scaffold. This is an extremely interesting strategy, as it presents potential to overcome normally encountered problems associated with porosity of the scaffold. Additionally, with particle-based systems shaped as scaffolds, the surface area for more chemical and biological reactions to take place is greatly increased.

The formation of 3-D scaffolds from materials in particulate form creates the potential for these systems to be used either in an acellular strategy (implanting of the scaffold and colonization of it by surrounding cells) or combining it with cells in vitro, creating a hybrid cell-material construct. Simultaneously, these scaffolds can also be used as delivery systems, having a multifunctional purpose – support and release of bioactive agents – enhancing the regenerative potential of the system.

3.1. Microparticle-based systems in 3-D scaffolds

Materials in particulate form in bone applications have as first examples the filling applications of ceramic particulate materials. Schepers et al. described the ability of bioactive glass particulates of narrow size range to act as filler for bone lesions. When implanted in the jaw of beagle dogs the particulates were capable of acting as nucleation sites for further bone repair, eliciting bone tissue formation throughout 5 mm defects in the beagle mandible as soon as 1 month after implantation.
However, as cells in the body grow in three dimensions anchored onto a network of extracellular matrix, a scaffold is needed to recreate the 3-D environment\textsuperscript{145}. Classical examples of materials shaped for bone tissue engineering involve 3-D porous structures obtained by conventional processing methods that, in an conductive approach, are implanted at an injury site and allow progenitor cells from the surrounding tissue to populate the wound site\textsuperscript{146}.

Given that porosity, pore size and interconnectivity are very important parameters for the success of a bone TE system, the strategy based on micron-size particles for fabrication of 3-D scaffolds seems to be promising, as a means of achieving more control over the above parameters. So far, the following strategies have been studied to fabricate scaffolds from materials in particulate form:

- Combining particulate materials with gels/glues.

In bone reconstruction, the combination of particulate ceramics and fibrin glue may result in the synergy of their properties, as the physical properties of the composite can be enhanced. The initial stability of the ceramic/fibrin glue composite may be achieved through its adaptation and adhesion to the walls of the bone defect. The biological properties might also be enhanced due to fibrin which acts positively on angiogenesis, cell attachment and proliferation\textsuperscript{147}. The problem associated with this type of approach is the lack of porosity. Although cell adhesion would be greatly enhanced by fibrin glue, the penetration of cells in the interior of the scaffold is limited by the lack of porosity.

- Dispersing microparticles within ceramic phases for posterior creation of porosity.

Other strategies have focused on dispersing microparticles within ceramic phases, where the rationale for this is that the microspheres will initially stabilize the graft but can then degrade to leave behind macropores on the calcium phosphate cement (CPC) for colonization by osteoblasts. The CPC matrix could then be resorbed and replaced with new bone\textsuperscript{148}. This relies on the degradation of the microparticles, which depends greatly on the material of which the microparticles are produced, as well as the implant site. It creates difficulties for osteoblast
colonization, particularly to the inner areas of the scaffold, as the particles might not degrade as fast as necessary to avoid the failure of the implant. An interesting way of overcoming these problems might be the incorporation within the matrix of the microparticles of enzymes that can degrade them and thus speed the process of pore-formation, as described by other researchers\textsuperscript{149,150}.

- Incorporating polymer microspheres with polymeric scaffolds.

This approach permits the incorporation of growth factor-containing polymeric microspheres during polymer scaffold fabrication\textsuperscript{151}. The basic principle of this approach is to transiently protect the microspheres with a water-soluble coating that resists the organic solvents used during scaffold fabrication. The incorporation of microspheres in scaffolds allows not only the protection of the growth factor during fabrication of the scaffold, but allows the scaffold to provide both structural support and controlled release properties.

- Sintering microspheres together.

The previous approaches have paved the way for the use of microparticles as scaffolds. Microparticles can be used to form three-dimensional scaffolds by utilizing the heating energy of a laser beam to sinter polymer microparticles allowing the fabrication of 3-D scaffolds with a controlled architecture and a fully interconnected network\textsuperscript{138,152,153}. By modifying processing parameters, such as sphere diameter and heating time, it is possible to tune the properties of the scaffold. It was found that increased microsphere diameter resulted in decreased modulus, as well as a positive correlation between sphere diameter and pore diameter\textsuperscript{154}. Heating time modifications showed that compressive modulus was dependent on the period of heating with longer heating times resulting in higher moduli, while the heating time not affecting the pore structure\textsuperscript{154}. These scaffolds can be further tested, not only in static but also in dynamic conditions such as the ones found in bioreactors.
3.2. Microparticle-based systems in hybrid cell-material constructs

Materials in particulate form have been used for combination with cells in two main approaches: the encapsulation of cells for site-specific delivery or the combination of scaffolds and cells in hybrid constructs in \textit{in vitro} approaches.

Examples of the former include the encapsulation of specific quantities of cells together with bioactive glass into alginate beads\textsuperscript{155}. Alginate beads have been extensively used for the encapsulation of several cell types\textsuperscript{156-162}. The study in question has shown that the encapsulated cells remained viable and secreted significantly more VEGF compared with beads containing no glass particles\textsuperscript{155}. This demonstrates that cells can be encapsulated for delivery and with the appropriate stimuli (here conferred by bioactive glass) can serve at the same time as the delivery vehicles for growth factors. With further optimization, this technique offers a novel delivery device for stimulating therapeutic angiogenesis, of which the lack in bone TE has been regarded a contributory factor for implant failure\textsuperscript{155}.

Temporary encapsulation of cells in microparticles may protect cells from short-term environmental effects such as those associated with the delivery to the regeneration site. To overcome certain problems encountered in cell therapy, particularly cell survival, lack of cell differentiation and integration in the host tissue, Tatard \textit{et al.}\textsuperscript{163} developed pharmacologically active microcarriers (PAM)\textsuperscript{163}. These biodegradable particles made with poly(D,L-lactic-coglycolic acid) (PLGA) and coated with adhesion molecules may serve as a support for cell culture and may be used as cell carriers presenting a controlled delivery of active protein\textsuperscript{163}. They can thus support the survival and differentiation of the transported cells as well as their microenvironment\textsuperscript{163}.

However, for bone applications, approaches that use the materials in particulate form not only to deliver and temporarily protect the cells seem to be more adequate, as they can also provide structural support while necessary. Ceramic materials, such as hydroxylapatite particles (both dense and microporous) have been evaluated both \textit{in vitro} and \textit{in vivo} as carriers in an injectable tissue engineered bone filler\textsuperscript{164}. After seeding and culturing goat mesenchymal progenitor cells on the different types of particles, several layers of cells and ECM held the particles together in a 3-D
arrangement. The subcutaneous implantation of the constructs (with individual particle size between 212-300 µm) in nude mice revealed abundant bone formation by 4 weeks\textsuperscript{164}.

An important issue in bone tissue engineering concerns the possibility of limited tissue ingrowth in tissue-engineered constructs because of insufficient nutrient transport\textsuperscript{145}. To overcome such limitations, Ducheyne and co-workers\textsuperscript{165-168} envisioned a strategy to use the HARV bioreactor and microcarriers to engineer constructs that can be used for bone tissue engineering purposes. In a first approach, the authors used bioactive glass and Cytodex-3 beads and rat stromal cells for assessing the feasibility of culture using a HARV bioreactor\textsuperscript{165}. It was observed that 3-dimensional multicellular aggregates consisting of multiple cell-covered Cytodex-3 microcarriers bridged together, as well as mineralization, and the expressions of alkaline phosphatase activity, collagen type I, and osteopontin were shown\textsuperscript{165}. The authors further developed bioactive ceramic hollow microspheres with an apparent density in the range 0.8-1.0 g cm\(^{-3}\) as microcarriers for 3-D bone tissue formation in rotating-wall vessels (RWV). Cell culture studies using rat bone marrow stromal cells and osteosarcoma cells showed that the cells attached to and formed 3-D aggregates with the hollow microspheres in a RWV. Extracellular matrix was observed in the aggregates\textsuperscript{166}. Similarly, polymer/glass/ceramic composite microspheres composed of modified bioactive glass (MBG) powders into a polylactic acid (PLA) matrix were shown to possess adequate properties for bone tissue engineering purposes\textsuperscript{168}. Other authors\textsuperscript{145} have used a similar approach, but mixing lighter-than-water (density, <1 g ml) and heavier-than-water (density, >1 g ml) microspheres of 85:15 poly(lactide-co-glycolide) and constructing the scaffold prior to cell seeding by sintering of the microspheres. When rat primary calvarial cells were cultured on the scaffolds in bioreactors for 7 days, the 3-D dynamic flow environment affected bone cell distribution and enhanced cell phenotypic expression and mineralized matrix synthesis within tissue-engineered constructs compared with static conditions\textsuperscript{145}. It has been found that with the stress stimulation inside the fluid in the RWV, the active expression of ALP can be increased and the formation of mineralized nodules can be accelerated\textsuperscript{169}. These works show that three-dimensional fabrication of engineered bone seems an adequate strategy.
3.3. Microparticle-based systems as scaffolds and carriers for bioactive molecules

By far the major field of application of particle-based systems (both in the micro and nano range) is as drug delivery systems, as described in detail in the first part of this review\textsuperscript{170}. Their small size but high surface area renders them attractive for a whole range of applications, including bone tissue engineering.

In bone tissue regeneration the use of conductive scaffolds in combination with the delivery of bioactive factors to direct cellular responses and subsequent tissue formation is a very attractive strategy to enhance regeneration\textsuperscript{146}, but parameters such as instability and rapid clearance (short plasma half-life) of these molecules after \textit{in vivo} bolus delivery have led to the need of advanced vehicles for localized release\textsuperscript{19,171,172}. The physicochemical properties of many peptides and proteins make their entrapment difficult, because inactivation is possible during their incorporation\textsuperscript{173}. Stability, solubility and sensitivity to light, heat, moisture and pH, intermolecular interactions following co-precipitation or gelling, adsorption and interaction with excipients are parameters which should be investigated in order to succeed in producing a stable association of peptides with particle-based systems\textsuperscript{173}. While encapsulation of peptides and small molecules into biodegradable microspheres can be achieved using several techniques and with different polymers, the encapsulation of proteins still poses major difficulties with respect to obtaining the ‘infusion-like’ or continuous release profiles with minimal initial burst and sufficient protein loading within the microspheres\textsuperscript{174,175}.

Drug delivery systems for bone applications has been mainly focused on 3-D porous scaffolds processed by conventional techniques, which present additional difficulties due to the possibility of destroying the bioactive agent. Some researchers have focused on the incorporation of microparticles loaded with bioactive agents into 3-D scaffolds, in an attempt to protect the bioactive agent and still maintain the 3-D structure of the scaffold, as described found by Mikos and co-workers, which have added poly(D,L-lactic-co-glycolic acid) / poly(ethylene glycol) (PLGA/PEG) microparticles loaded with the osteogenic peptide TP508 to a mixture of
poly(propylene fumarate) (PPF), poly(propylene fumarate)–diacrylate (PPF–DA), and sodium chloride (NaCl) for the fabrication of PPF composite scaffolds that could allow for tissue ingrowth as well as for the controlled release of TP508 when implanted in an orthopedic defect site. Other authors have used a three-dimensional chitosan scaffold, which was combined with transforming growth factor-beta1 (TGF-β1)-loaded chitosan microspheres.

However, the incorporation of bioactive agents into micron size systems and using them simultaneously as scaffolds and release systems seems an extremely interesting alternative. Examples include the use of dextran-derived materials, which possess hydrophilic properties and ability to control drug dissolution and permeability. Dextran-glycidylmethacrylate (Dex–GMA)/poly(ethylene glycol) (PEG) microspheres with entrapped recombinant human bone morphogenetic protein-2 (rhBMP-2) showed full preservation of its biological activity. rhBMP-2 microspheres have good biological effects on cultured periodontal ligament cells, and could achieve a longer action time than concentration of rhBMP-2 solution. These properties make those microspheres interesting osteoconductive BMP carriers, allowing to decrease the amount of implanted factor required for tissue regeneration. Similarly to BMPs, Insulin-like growth factor I (IGF-I) exerts an important role during skeletal growth and bone formation. Therefore, its localized delivery appears attractive for the treatment of bone defects. To prolong IGF-I delivery, this molecule was entrapped into biodegradable poly(lactide-co-glycolide) microspheres and the system evaluated in two defect models of ovine long bones, a metaphyseal drill hole and a segmental tibia defect. New bone formation was observed within 3 weeks in the drill hole and bridging of the segmental defect within 8 weeks. The authors have shown that the IGF-I delivery system downregulated inflammatory marker gene expression at the site of bone injury, induced new bone formation and reduced bone resorption.

Other approaches try to gather further properties within a single system, as the one where in situ-hardening composites are formed based on an alginate hydrogel matrix formulated with β-TCP granules and poly(lactide-co-glycolide) microspheres loaded with the osteoinductive growth factor insulin-like growth factor I (IGF-I). This approach combines release properties, structural support and a ceramic material with osteoconductive properties for enhanced bone formation.
regeneration. Materials such as collagen/chitosan composite microgranules were fabricated as bone substitutes for the purpose of obtaining high bone-forming efficacy. The microgranules have the flexibility to fill various types of defect sites with closer packing. The interconnected pores formed spaces between the microgranules, which allowed new bone ingrowth and vascularization. In addition, TGF-β1 was incorporated into the microgranules in order to improve bone-healing efficacy. The TGF-β1-loaded microgranules demonstrated a higher bone regenerative capacity in the rabbit calvarial defects after 4 weeks than the TGF-β1-unloaded microgranules.

6. CONCLUSIONS

Bone repair has been the subject of intensive research. Approaches in clinical use aim the regain of function, using materials that replace the damages tissue rather than regenerating it. Currently, the approach of research regarding bone TE is to induce regeneration rather than just functional repair. As so, tissue engineering can now simply defined as the “science of persuading the body to heal by its intrinsic repair mechanisms”.

The complexity of skeletal tissues has been hindering the development of an effective regeneration system. Nevertheless, huge steps are being taken regarding the use of progenitor/stem cells, adequate scaffold materials and growth factors/bioactive agents. The combination in a single system of such properties – structural support, cell support and controlled release – is the way to go. And materials in the particulate form have all the potential needed for achieving such goal.
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CHAPTER II
Chapter II

Materials and Methods

The materials used in the studies described in the present thesis were the following:

A. Starch-based materials

Starch-based materials were used for the production of microparticles. Three different blends of cornstarch with synthetic polymers were selected, in order to combine the best properties of each of the materials.

These blend materials were supplied by Novamont (Novara, Italy) and ground to a fine powder at the facilities of the Department of Polymer Engineering, University of Minho.

Blends of cornstarch:

- Starch and polylactic acid 50/50 (wt%), with the designation of SPLA;
- Starch and ethylene vinyl alcohol 50/50 (wt%), with the designation of SEVA-C;
- Starch and cellulose acetate 50/50 (wt%), with the designation of SCA.

Potato starch:

Additionally, other starches were used, such as starch from potato origin. Two different types were tested: both native and modified to render the starch soluble in water.

- Native potato starch
- Modified potato starch, with two different degrees of esterification (DE): DE=2 and DE=20, with the designation of Paselli 2 (Pa2).
These materials were supplied by ATO (Wageningen, Netherlands) as a powder.

**Commercial starch:**

Besides starch-based materials from corn and potato, two commercial starches were also selected for producing micron-size systems. These starches were purchased from Sigma (St. Louis, USA)

- Soluble starch, ACS reagent, S9765.
- Starch from corn, S4126.

**B. Polylactic acid** [Poly D,L lactic acid (PLA)] with a molecular weight (Mw) of 50000, was supplied by Polysciences, Warrington, USA.

**C. Hydroxylapatite** (HA), non-sintered (in house produced).

**D. Bioactive Glass 45S5** (46.1% SiO₂, 24.4% Na₂O, 26.9% CaO, 2.6% P₂O₅, mole%) was ground before use to a particle size smaller than 60 µm and 16 µm. This material was supplied by MO-SCI (Rolla, MO, USA).

The development and characterization of starch-based microparticles consisted on the following sections:

1. Development of microparticles – in this section we made use of several materials and methods to produce starch-based microparticles;

2. Characterization of the developed microparticles – the characterization consisted on the analysis of the morphology, size distribution, degradation and water uptake, bioactivity and cytotoxicity;
3. Interaction of starch-based microparticles with cells from the osteoblastic lineage – screening of the effect of starch-based microparticles over cells of the osteoblastic lineage (both a cell line and primary cultures) and

4. Incorporation and release of biologically active molecules using starch-based microparticles – evaluation of the ability of the developed systems to serve as carriers for bioactive molecules, such as anti-inflammatory agents and growth factors.

1. DEVELOPMENT OF MICROPARTICLES

For the development of starch-based microparticles, combinations of the materials referred above and different methods of production were tested to produce a variety of systems. This aimed to generate a diversified set of materials with a wide array of properties. This pool of materials would allow the selection the most promising materials to be evaluated further for applications such as drug delivery to bone defect sites.

1.1. Emulsion crosslinking

This method has been previously developed in the 3B’s Research Group\textsuperscript{1}. In the present work some modifications have been introduced to the previously reported methodology. 10 g of starch-based material were dissolved in 50 ml of deionized (DI) water, and 2 g of a crosslinking agent, trisodium trimetaphosphate (TSTP) (Sigma, USA) were dissolved in the starch solution. This solution was then emulsified in 50 ml of paraffin oil (Fluka) containing 2.5 g of Span 80 (Fluka), a hydrophobic surfactant, used to promote emulsification between the two phases. After this, 2.5 ml of NaOH 160 g/L were added dropwise, and the reaction was allowed to proceed for 6 hours or until the particles are formed. The particles were then neutralized with a 1:1 solution of HCl 37\%
and water. The particles were further washed with DI water. According to their size, centrifugation could be used for washing. The particles were freeze-dried or stored in DI water containing 0.1% of sodium azide (Sigma, USA) to prevent bacterial contamination.

To obtain composite microparticles, the ceramic phase was added to the polymer (in solution) and a homogeneous suspension was formed before proceeding with the above-described method.

The following ratios were tested:

- 50% of the polymer weight of hydroxylapatite
- 20% of the polymer weight of Bioactive Glass 45S5 and
- 30% of the polymer weight of Bioactive Glass 45S5.

### 1.2. Solvent evaporation/emulsion crosslinking

#### 1.2.1. TSTP as crosslinking agent

The method for producing starch microparticles was based on a solvent-evaporation method. The starch-based material was dissolved in DI water or dimethyl sulfoxide (DMSO), according to the solubility of the starch-based material at a ratio of 1 g starch/10 ml of solvent. When complete or partial dissolution was achieved, 0.5 g of TSTP was dissolved in the starch solution. When the crosslinking agent was dissolved, 0.1 ml of Tween 80 (Sigma, USA) was added and allowed to dissolve. 20 ml of cyclohexane (Sigma, USA) were added, and the emulsion allowed to form, covered by aluminum foil to prevent cyclohexane evaporation. The time required for the formation of the emulsion varies, but did not exceed 30 minutes. After the formation of the emulsion, 1 ml of NaOH 160 g/L was added in 10 times, dropwise. The reaction proceeded for 6 hours or until formation of the particles. The emulsion was uncovered and the cyclohexane allowed evaporating
overnight. After the complete evaporation, the particles are washed with DI water and stored in the refrigerator in DI water with 0.1% of sodium azide.

To obtain composite microparticles, the ceramic phase was added to the polymer (in solution) and a homogeneous suspension was formed before proceeding with the method. The following condition was used: 50% of the polymer weight of hydroxylapatite.

1.2.2. Epichlorohydrin as crosslinking agent

In an attempt to reduce the reaction time by reducing the time necessary for crosslinking to occur, epichlorohydrin was used instead of TSTP. The former has been used in the literature\textsuperscript{2,3} as a very effective crosslinking agent.

The method for producing starch microparticles crosslinked with epichlorohydrin was basically the same as described for the solvent evaporation method using TSTP as crosslinking agent. The starch-based material was dissolved at a ratio of 1 g starch/10 ml of a solution of 2M of NaOH (in water) or dimethyl sulfoxide, according to the solubility of the starch-based material. When the material was dissolved, 0.1 ml of Tween 80 was added and allowed to dissolve. 20 ml of cyclohexane are then added, and the emulsion was allowed to form, covered by aluminum foil to prevent cyclohexane evaporation. The time required for the formation of the emulsion varies, but does not exceed 30 minutes. After the formation of the emulsion, the reaction vessel was transferred to a thermostatic bath set previously at 40\textdegree C. After the stabilization of the temperature, 2 ml of epichlorohydrin (Sigma, USA) are added dropwise under strong stirring to prevent aggregation. The reaction was allowed to proceed for 6 hours, or until formation of the particles. After the formation of the particles the reaction vessel was placed at room temperature and was uncovered to allow the cyclohexane to evaporate overnight. After the complete evaporation, the particles are washed with DI water and stored in the refrigerator in DI water with 0.1% of sodium azide.
To obtain composite microparticles, the ceramic phase was added to the polymer (in solution) and a homogeneous suspension was formed before proceeding with the method. The following ratio was used: 50% of the polymer weight of hydroxylapatite.

1.3. Solvent evaporation

The solvent method was described by Q. Qiu et al.\textsuperscript{4}, and briefly, 0.8 g of PLA were dissolved in 5 ml of methylene chloride with magnetic stirring. After complete dissolution, the solution was dispersed in 200 ml of a 0.5% PVA (Poly Vinyl Alcohol) solution with high-speed agitation using a Caframo dual-range stirrer (Fisher Scientific, USA) at a speed of 660 rpm/min. The particles were then hardened for 4 hours, after which they were washed and filtered.

The solvent evaporation method used to produce starch-based microparticles was based on the method to produce PLA microparticles described in Q. Qiu et al.\textsuperscript{4}, slightly modified as follows. In brief, 1 g of the starch-based material was dissolved in 10 ml of methylene chloride (Sigma, USA) under magnetic stirring. After complete dissolution, the solution was dispersed in 200 ml of a 0.5% PVA (Poly Vinyl Alcohol) (PolySciences, USA) solution with high-speed agitation using a Caframo dual-range stirrer (Fisher Scientific, USA) at a speed of 660 rpm/min. The particles were then hardened for 3.5 hours, after which they were washed and dried. They were separated according to their size in a sonic sieve.

To obtain composite microparticles, the ceramic phase was added to the polymer (in solution) and a homogeneous suspension was formed before proceeding with the above-described method. The following condition was used: 30% of the polymer weight of Bioactive Glass 45S5. Table I summarizes the conditions tested for the development of microparticles and that were used to choose the conditions for further testing.
Table I. Summary of materials and methods used for the development of microparticles. Y – method tested; N - method not tested; (+) microparticles formed; (-) no microparticles formed. HA – hydroxyapatite; BG – Bioactive Glass 45S5; EpiHCl – epichlorohydrin.

<table>
<thead>
<tr>
<th>Materials</th>
<th>TSTP</th>
<th>EpiHCl</th>
<th>Emulsion Crosslinking</th>
<th>Solvent Evaporation</th>
<th>Solvent Emulsion</th>
<th>Solvent Emulsion (modified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEVA-C</td>
<td>Y(+)</td>
<td>Y(-)</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>SCA</td>
<td>Y(+)</td>
<td>Y(-)</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>SPLA</td>
<td>SPLA</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y(+)</td>
</tr>
<tr>
<td></td>
<td>SPLA+BG</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y(+)</td>
</tr>
<tr>
<td>Native</td>
<td>NP</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Potato</td>
<td>NP+ HA</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Soluble</td>
<td>Pa2 DE=2</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>N</td>
</tr>
<tr>
<td>Potato</td>
<td>Pa2 DE=20</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Pa2 DE=2 + HA</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Pa2 DE=20 + HA</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>Y(+)</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Pa2 DE=2 + BG</td>
<td>N</td>
<td>N</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>Pa2 DE=20 + BG</td>
<td>N</td>
<td>N</td>
<td>Y(+)</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Soluble</td>
<td>PLA</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Y(+)</td>
<td>N</td>
</tr>
</tbody>
</table>

As observed in Table I, it was possible to obtain microparticles by means of using several different conditions. Both polymer-based and composite microparticles could be produced. At this
point it was necessary to perform a selection of the conditions for further studies, having into account the following parameters:

- **Morphology** of the resultant microparticles – the ideal morphology was considered to be spherical.
- **Stability** of the microparticles – the maintenance of the integrity/shape of particles in a solution at room temperature and physiological temperature (37°C) was necessary considering the possible applications.
- **Methodology** (time of preparation, solvents) – shorter time for the onset of the method, easiness of the method, use of non-toxic solvents
- **Time of synthesis** and **scalability** - reduced time of production was considered an advantage, as well as the possibility of producing larger amounts of material within one experiment.
- **Yield** - ratio of material converted into microparticles/initial amount of raw material.

Having into account the above-mentioned parameters, all the conditions were classified, and the conditions that with the best score were chosen and are as follows:

- SPLA microparticles obtained by the modified solvent evaporation/emulsion method;
- SPLA/BG 45S5 microparticles obtained by the modified solvent evaporation/emulsion method;
- Pa2 DE=2 microparticles obtained by the emulsion crosslinking method using TSTP as crosslinking agent and
- Pa2 DE=2-BG 45S5 microparticles obtained by the emulsion crosslinking method using TSTP as crosslinking agent.
2. CHARACTERIZATION OF THE DEVELOPED MICROPARTICLES

The characterization of the developed microparticles was divided into three major aspects:

- General characterization of the properties of the materials focusing on the morphology, size distribution, degradation and water uptake;
- Assessment of the bioactivity of materials, by formation of a calcium phosphate layer at the surface of the materials when immersed in a simulated body fluid and
- Short-term cytotoxicity screening by indirect contact, a necessary step for materials that are to be studied in direct contact with cell populations.

2.1. Morphological Characterization

The microparticles were morphologically characterized by light microscopy and scanning electron microscopy (SEM). The microparticles were observed and photographed in an optical microscope with an image acquisition system (high resolution color video camera and Image-Pro Plus analysis software, Media Cybernetics).

SEM analysis was performed on samples gold sputter-coated or carbon sputter-coated and analyzed using a Leica Cambridge S-360 model or a Jeol 6300F. Voltage varied between 8 and 10 kV, and magnifications ranged between 100-10000 times.

2.2. Size distribution

The distribution of sizes within each batch of produced microparticles was determined by sieving microparticles in a sonic sifter (ATM sonic sifter, model L3P, ATM Co.) using sifts with
the following sizes: 20, 40, 70, 104, 145, 210 and 350 µm. The size distribution was assessed by the weight of each of the sifted fractions.

2.3. Degradation & water uptake

Weight loss and the water uptake are two key parameters in the evaluation of biodegradable materials. Weight loss and water uptake translate into the degradation behavior and the capability of the materials to incorporate water in their matrix (swelling), respectively. These two parameters might allow predicting the behavior of a given material when inserted in the body and subjected to physiological fluids.

For the determination of the degradation and water uptake profiles, microparticles were weighted and immersed in a saline phosphate buffer solution (PBS) at physiological pH (pH 7.4) at a ratio of 1mg/1ml, and placed into a water bath at 37ºC with constant agitation (60 rpm). After pre-determined time periods (up to 30 days for water uptake, 90 days for weight loss) the vials containing the immersed microparticles were centrifuged at low speed for particle deposition and the supernatant was removed for further measurements of degradation products. The remaining wet microparticles were weighted for water uptake measurements. Water uptake was determined using equation 1:

\[
\% \text{ Water uptake} = \left(\frac{m_w - m_i}{m_i}\right) \times 100 \quad \text{ (equation 1)}
\]

where \(m_i\) is the initial weight and \(m_w\) is the wet weight of the sample. After each time period the samples were dried at 37ºC and the final mass of the samples weighted to determine the weight loss according to the following equation:

\[
\% \text{ Weight loss} = \left(\frac{m_f - m_i}{m_i}\right) \times 100 \quad \text{ (equation 2)}
\]
where \( m_f \) is the final weight of the dry sample and \( m_i \) is the initial weight of the sample. Triplicates were performed for each sample at each time period and the results are shown as mean ± standard deviation.

Samples were analyzed for their morphology (by SEM, as described in 2.1.) at the end of the weight loss studies.

2.4. In vitro bioactivity

In vitro bioactivity of a material is evaluated by its ability to form a calcium-phosphate (Ca-P) layer at its surface when immersed in a solution simulating the human blood plasma ionic composition\(^6\). The use of this solution was first described by Kokubo et al.\(^7\).

The following chemicals were used for preparing the simulated body solution, a Tris buffer complemented with electrolytes: 0.05 M Tris buffer solution, Trizma-HCl, sodium chloride (NaCl), potassium chloride (KCl), sodium hydrogen carbonate (NaHCO\(_3\)), magnesium chloride hexahydrated (MgCl\(_2\).6H\(_2\)O), magnesium sulphate heptahydrated (MgSO\(_4\).7H\(_2\)O), dipotassium hydrogen phosphate anhydrous (K\(_2\)HPO\(_4\)), calcium chloride anhydrous (CaCl\(_2\)), used as received. Table II contains the concentrations of each of the ions in the TE solution compared to the values of the same ions in the blood plasma.
Table II. Chemicals and respective concentrations used to prepare the Tris solution with electrolytes.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Amount (g/L)</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M Tris solution</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trizma®-HCl</td>
<td>5.1</td>
<td>32,36</td>
</tr>
<tr>
<td>NaCl</td>
<td>7.3050</td>
<td>125</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2336</td>
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</tr>
<tr>
<td>NaHCO₃</td>
<td>2.3683</td>
<td>22.8</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>0.1233</td>
<td>0.607</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.2033</td>
<td>0.825</td>
</tr>
<tr>
<td>K₂HPO₄ (anhydrous)</td>
<td>0.2742</td>
<td>1.57</td>
</tr>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>0.2773</td>
<td>2.5</td>
</tr>
</tbody>
</table>

2.4.1. Immersion in Tris buffer with electrolytes

Starch-based microparticles were immersed for in vitro testing of their bioactivity in a 0.05 M tris hydroxymethyl aminomethane-buffered solution complemented with electrolytes typical for plasma. The use of this solution was described previously⁶,⁸, and it was used to obtain pH 7.4 at 37°C without further need of pH adjustment. Starch-based particles were immersed at two different ratios: 5 mg/ml for polymeric microparticles and 3 mg/ml for composite microparticles, in a CO₂ incubator at 37°C. Different ratios for polymer and composite microparticles were used based on the ratio previously used for immersion of Bioactive Glass particles, which was 1 mg/ml⁹. For composite particles, immersion at 3 mg/ml was chosen since the reinforcement of the polymer particles with Bioactive Glass 45S5 had a value of 30%. For polymeric particles, due to the absence of ceramic phase (BG 45S5) the probability of these materials to be bioactive was reduced or null, the rate was increased to 5 mg/ml.
The vials were placed on an orbital shaker at a rotational speed of 150 rpm for up to 21 days without solution exchange. At designed time points (1, 2, 3, 7, 10, 14 and 21 days), the samples were removed from the shaker, the pH was measured and the microparticles separated from the solution by filtration. The later were then dried and stored in a desiccator until further analysis.

2.4.2. Post immersion solution analysis

The post immersion solutions were used to perform chemical analysis, namely for changes in Ca, Si and P concentrations. When a Ca-P layer is formed at the surface of a material containing Si, there is an increase in Si in the solution and a depletion of both Ca and P. Analyzing these elements in the solution allows one to infer about the presence of the Ca-P layer.

Ca and Si concentrations were measured by Atomic Absorption Spectrophotometry. For the Ca measurements a solution of 1% LaCl₃ was used, while the Si measurements were performed directly from the post immersion solution. For the P measurements a UV-Visible spectrophotometer was used at 400 nm and a colorimetric method was employed.¹⁰,¹¹

The phosphate concentration was measured as a phosphomolybdenate complex (molybdenum yellow) in a UV-Visible spectrophotometer at 400 nm. As every spectroscopic method, this technique relies on the absorption of electromagnetic radiation (ultraviolet) by matter, and the Beer-Lambert law applies similarly. A linear relationship can be achieved between absorbance and concentration in a region of concentration from 1 to 40 mg/L with correlation coefficient of 1.

A solution of ammonium molybdate (1 volume of ammonium molibdate, 1 volume of H₂SO₄ 5N and 2 volumes of acetone) was prepared. To measure the absorbance of each sample, 0.4 ml of sample was combined with 1.5 ml of the solution described above and with 0.1 ml of citric acid. Proper calibration was performed with phosphate standards in the range between 0 and 30 ppm.

Samples were also analyzed by ICP-OES (Inductively Coupled Plasma - Optical Emission Spectroscopy) at the University of Aveiro, Portugal.
2.4.3. Surface analysis

Surface analysis was performed using Fourier Transform InfraRed Attenuated reflection (FTIR-ATR), Scanning Electron Microscopy/Energy Dispersive X-Ray Spectroscopy (SEM/EDS) analysis and X-Ray Diffraction (XRD) analysis.

Scanning Electron Microscopy (SEM)/Energy Dispersive Spectroscopy (EDS) was performed on carbon sputter-coated samples to analyze the elemental composition of the surface of the material, for the presence of Ca and P and their respective ratio. Fourier Transformed InfraRed Attenuated reflection (FTIR-ATR) was employed to evaluate the presence of characteristic groups pertaining to a Ca-P layer formed at the surface of the microparticles. FTIR-ATR spectra were recorded at least at 32 scans with a resolution of 2 cm\(^{-1}\) in a FTIR spectrophotometer (Perkin-Elmer 1600 Series). All the samples were analyzed using a single reflection ATR system (MKII Golden Gate\textsuperscript{TM}, Specac) with a diamond crystal (angle of incidence = 45°; active sampling area 0.8×0.8mm; depth of penetration 20 µm).

X-Ray Diffraction was used to analyze the presence of a Ca-P layer covering the microparticles after the in vitro bioactivity studies for its crystalline nature, using an X’Pert PW3040 (Philips) apparatus and the 2θ scan method.

2.5. Short-term cytotoxicity screening

The first step in the assessment of the biocompatibility of a material is the screening of its cytotoxicity. For this initial test, leachables from the materials and animal cells in culture are generally used. The procedure follows the guidelines from ISO (International Standards Organization) for testing of biomedical materials, and consists on an indirect contact test i.e., it is
not the material itself that is placed in contact with the cell population, rather leachables from the material (since these are degradable materials).

For the screening of the cytotoxicity by indirect contact with the materials, two different tests were performed: MTT and total protein quantification test. These two methods allow quantifying the effect of a material over the viability and proliferation of a cell culture, and thus infer about the cytotoxic behavior (or lack of) of a material.

The cells used in this study were an immortalized cell line of subcutaneous areolar fibroblasts from mouse origin purchased from Collection of Cell Culture (ECACC, UK, ref. 85011425), designated L929. The cells were grown in monolayer systems in Dulbecco’s Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS), 1% of antibiotics and used between passages 6 and 12.

2.5.1. MTT

The MTT test is a biochemical test widely used to assess cytotoxicity by measuring cell viability and proliferation in a qualitative way\textsuperscript{12,13}. This biochemical test is based in the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide), (which is water-soluble and has a yellow tonality) by the cell mitochondrial enzyme succinate dehydrogenase, yielding a purple color salt insoluble in water\textsuperscript{14}. The salt absorbs at a wavelength of 570 nm and since only living cells have the capability of metabolize the MTT, it gives a measurement of the viable cells\textsuperscript{14}.

The procedure followed for this test corresponds to the ISO MEM Elution test for short-term cytotoxicity assessment.

Starch-based microparticles were placed in conic tubes with DMEM culture medium and placed under constant shaking (60 rpm) at 37ºC for 24 hours for the materials to release eventual leachables (formation of extracts from the materials). An extraction control was performed, which consists on DMEM culture medium, placed in the same conditions as the samples. This control was performed to assure that the extraction conditions by themselves do not alter the properties of
the culture medium. After 24 hours the extracts were filtered through a 0.45 μm pore size filter, placed in contact with a cells monolayer with 80-90% confluence and further incubated for 72h. After this time period the viability of the cells is evaluated by the MTT test as described below.

A MTT (Sigma, St. Louis, USA) solution of 1mg/ml in DMEM culture medium (supplemented as described above) was added to each well. The plate is then incubated at 37ºC, in a humidified atmosphere for 4 hours, after which an isopropanol/HCl solution is added to each well and further incubated for 15 minutes, in order to enhance the dissolution of the formazan crystals. The optical densities at 570 nm and 650 nm (background) were read on a multiwell plate reader (Molecular Dynamics, Amersham, USA) against a blank of MTT solution and isopropanol.

All the materials were tested in 10 replicates for each extract for at least two independent experiments with reproducible results. The results are expressed as a percentage of the control (scored as 100% viability) as mean ± standard errors.

2.5.2. Total Protein Quantification

Total protein is generally assayed as a measurement of cell proliferation. The method that was used to quantify the total protein uses the Micro BCA Protein Assay Reagent Kit, in which bicinchoninic acid (BCA) is the detection reagent for a Cu complex, which is formed when Cu²⁺ is reduced by proteins in an alkaline environment. The purple color product is due to the chelation of two molecules of BCA with one Cu ion. This complex is water-soluble and absorbs at 562 nm, and its optical density is linearly correlated with protein concentration.

The procedure followed is as described for the MTT test. After the 72 hours’ incubation, the cells are washed with PBS and the BCA reagent is added. After an incubation period, the optical densities were measured. Total protein (in μg) was determined using a BSA standard curve.

All the materials were tested in 10 replicates for each extract for at least three separate experiments, with reproducible results. The results are expressed as percentage of the control (scored as 100% viability) as mean ± standard errors.
3. INTERACTION OF STARCH-BASED MICROPARTICLES WITH CELLS FROM THE OSTEOBLASTIC LINEAGE

In order to assess the effect of starch-based microparticles (both polymer and composite) over cells from the osteoblastic lineage, two different types of cells from the osteoblastic lineage were used: rat bone marrow stromal cells (RBMCs) and MC3T3-E1 cells, a pre-osteoblastic cell line from mouse calvaria.

RBMCs were freshly isolated from rat femurs and initially used for these studies. However, in order to have a more controlled system to evaluate the performance of starch-based microparticles, it was decided to use a well-characterized cell line, MC3T3-E1 pre-osteoblastic cells.

The overall procedure consisted on the following:

i. Evaluation of the number of cells adhered to the surface of starch-based microparticles;
ii. Assessment of the viability and proliferation of adhered cells and
iii. Expression of the osteoblastic phenotype by cells at the surface of the microparticles.

3.1. Cell sources

3.1.1. Rat bone marrow stromal cells

These cells have been shown to differentiate into osteoblasts in vitro when cultured with the appropriate supplements. To evaluate if this same behavior could still be observed when cultured at the surface of starch-based microparticles, these cells were isolated and cultured according to the method described by Maniotopoulos et al.\textsuperscript{16}, as detailed below.

After sacrificing 5-week old male Wistar rats, the femurs were removed, cleaned from soft tissue and immersed in 70% ethanol very briefly. The epiphyses were then cut of and discarded,
while the diaphyses were flushed with a syringe into α-MEM culture medium supplemented with 10% FBS, 1% antibiotics (50 µg/ml gentamicin, 100 µg/ml ampicillin, 0.3 µg/ml fungizone), ascorbic acid, 10 mM β-glycerophosphate and 10⁻⁸ M dexamethasone. The isolated cells were then cultured in standard tissue culture conditions (37°C, 5% CO₂) for 6 days, with periodic media changes for removing debris (unattached cells, mainly from the hematopoietic lineage).

3.1.2. MC3T3-E1 cells

Cell lines have been established in order to have a more controlled system compared with primary cell cultures. The use of cell lines is a resource that allows to obviate the variability associated to the donor and to have a defined cell population. For this reason, cell culture studies at the surface of starch-based microparticles made use of an established cell line of the osteoblastic lineage. MC3T3-E1 subclone 4 cells are a preosteoblastic cell line derived from newborn mouse calvaria. The cells, obtained from ATCC (CRL-2593), have fibroblastic morphology, and this subclone exhibit high levels of osteoblast differentiation. The cells were cultured in DMEM medium supplemented with 10% FBS and 1% of antibiotics (penicillin – streptomycin) and cultivated in standard tissue culture conditions (37°C, 5% CO₂).

3.2. Cell adhesion efficiency

A study of the adhesion efficiency of cells to the surface of the different types of microparticles was necessary to further understand the results. For this purpose, a simple method for determining the adhesion efficiency was devised.

The ability of MC3T3-E1 cells to adhere to the surface of starch-based microparticles was evaluated for up to 6 h. This time period was chosen considering that these are anchorage
dependent cells and therefore their viability depends on their efficient attachment to a surface, which for these cells has been found to occur up to two hours in TCPS.

In order to determine the adhesion efficiency, 1 µl volume of microparticles was added to a suspension of 2x10^5 cells. The cells were allowed to adhere for 30 min, 1, 2 and 6 h. After each time period, the microparticles were washed to make them free of non-adherent cells and transferred to a new vial. A volume of trypsin, enough to cover the microparticles, was added to detach the adherent cells, which was then neutralized by the addition of complete culture medium. Cells were then counted in a Neubauer chamber. Adhesion efficiency was determined as the percentage of adhered cells versus the total number of cells seeded. Data reports results from 3 independent experiments.

3.3. Cell seeding and culture at the surface of starch-based microparticles

A pre-determined amount of starch-based microparticles enough to cover the bottom of a well of a 24-well plate, was weighted and placed in non-adherent cell culture plates. The use of this particular type of culture plates was to prevent the adhesion of cells to the surface of well and thus mask the results from cells adhered to the surface of the starch-based microparticles. Prior to cell seeding, the materials were ethanol-sterilized and pre-incubated for 24 hours in 500 µl of culture medium in the 24-well plates.

RBMCS in suspension were dropped over the microparticles at a density of 5x10^4 cells/well. Two studies were performed – both polymer and composite materials were cultured in two different conditions, culture medium supplemented with and without dexamethasone. Dexamethasone was used – together with ascorbic acid – as a means of inducing the commitment of rat bone marrow cells into the osteoblastic lineage\(^{10}\). The absence of dexamethasone in one set of experiments aimed to assess if cells cultured on the surface of starch-based particles could commit to the osteoblastic lineage even in the absence of dexamethasone.
The cultures were maintained for 17 days, with periodic evaluations whenever the culture medium was changed (every three days).

During the course of the studies with rat bone marrow cells it came out that it was necessary to further optimize the cell culture method.

Therefore, the method of seeding MC3T3-E1 cells on the surface of starch-based microparticles was different from the one utilized for RBMCs. Instead of dropping the cell suspension over the particles resting at the bottom of a non-adherent tissue culture well, the microparticles and cells were mixed in a tube and after a suitable period transferred to cell strainers inserted into tissue culture wells.

A volume of 10 µl of microparticles was added to a suspension of 5x10⁵ MC3T3-E1 cells. The microparticles and cells were mixed and centrifuged for 30 s at 100 rpm. After a maximum period of 12 h, the microparticles with seeded cells were transferred to 6-well plates containing 40 µm pore size cell strainers (BD Falcon, Bedford, MA, USA). The cell strainers prevented particle loss during culture medium changes and also allowed unattached cells to be washed away, therefore preventing the quantification of non-viable cells. The cultures were maintained for 14 days.

3.4. Viability assessment using confocal laser microscopy

Viability of MC3T3-E1 cells adhered to starch-based microparticles was assessed by confocal laser microscopy (Inverted Confocal Microscope, Olympus FloView, Melville, NY, USA). For this purpose was used a viability fluorescent dye – CellTracker Green CMFDA (Molecular Probes, Eugene, OR, USA). This dye diffuses through the cell membranes and once inside the cell, the CellTracker, containing a chloromethyl group that reacts with thiols, is transformed into a cell-impermeant fluorescent dye-thioether adduct. Only living cells have the ability to allow this reaction to occur, and this principle was used to determine the distribution and viability of cells adhered to the surface of starch-based microparticles.
The culture medium from the samples was replaced by a 1:1000 dilution of CellTracker in serum-free DMEM. After 30 min the working solution was removed and replaced with complete culture medium. After a second 30 min incubation period, the samples were analyzed in a laser confocal microscope, with an excitation laser of 517 nm. Images of the samples were obtained by stacking of 20 μm planar slices.

3.5. Cell proliferation at the surface of starch-based microparticles

Cell proliferation was evaluated using either the total protein quantification (as described in section 3.1.2), and by quantifying DNA content, which was measured using the PicoGreen dsDNA kit (Molecular Probes, USA). PicoGreen dsDNA reagent is an ultra sensitive fluorescent nucleic acid stain for the quantification of double stranded DNA (dsDNA) in solution. At each time point in culture, cell strainers were removed and the contents (particles with cells) washed with isotonic saline solution and centrifuged. A minimal volume of 0.1 N NaOH was added to release DNA from the cells. An aliquot of the DNA suspension was added to 10x Tris-EDTA buffer, to which was then added the PicoGreen reagent (previously prepared in 10x Tris-EDTA buffer) in a 1:1 vol:vol, and fluorescence measured in a microplate reader at 485 and 535 nm excitation and emission wavelengths, respectively. Lambda DNA was used as standard. The data presents results of at least three independent experiments.

3.6. Alkaline Phosphatase activity

Alkaline phosphatase hydrolyses phosphate esters and is involved in the initial processes of bone extracellular matrix mineralization. Consequently, it is widely regarded as an indicator of osteoblastic lineage and analyzed by tests based on the hydrolysis of substrates containing
phosphate. It is a membrane-bound enzyme, being also secreted to the extracellular milieu. Based on this fact, its activity was analyzed in the supernatant (culture medium) of the samples (RBMCs cultured at the surface of the particles). The activity of the secreted enzyme was quantified by the specific conversion of p-nitrophenyl phosphate (pNPP) into p-nitrophenol (pNP)\textsuperscript{19}. Supernatants were collected at each time point and frozen at -20\textdegree{} C until further analysis. The enzyme reaction was set up by mixing 50 \(\mu l\) of the sample with 150 \(\mu l\) of substrate buffer containing 1 M diethanolamine HCl (pH 9.8) and 2 mg/ml of pNPP. The solution was incubated at 37\textdegree{} C for 1 hour and the reaction was then stopped by a solution containing 2 M NaOH and 0.2 mM EDTA in distilled water. The optical density was determined at 405 nm with a reference filter at 620 nm. A standard curve was made using pNP values ranging from 0 to 200 nmol/ml. The results are expressed in nmol of pNP produced/ml/min.

In the above described, it was quantified the activity of the enzyme alkaline phosphatase secreted to the culture medium. However, quantifying the membrane-bound enzyme is much more accurate since it is only quantified the enzyme present in cells viable at the moment of the test and not the secreted enzyme accumulated in the culture medium. Alkaline phosphatase was measured using the AttoPhos AP fluorescent Substrate System (Promega, Madison, WI, USA), which shares the same principle as the method described above. AttoPhos Substrate (2\textprime{}-[2-benzothiazoyl]-6\textprime{}-hydroxybezothiazole phosphate [BBTP]) is cleaved by alkaline phosphatase to produce inorganic phosphate (P\textsubscript{i}) and the alcohol, 2\textprime{}-[2-benzothiazoyl]-6\textprime{}-hydroxybenzothiazole (BBT). This enzyme-catalyzed conversion of the phosphate form of AttoPhos Substrate to BBT is accompanied by an enhancement in fluorescence. Samples (microparticles and adhered cells) were transferred to a 1.5 ml tube, centrifuged at 14000 rpm for 1 min, the supernatant (culture medium) was discarded and the pellet was suspended in 1ml of ice-cold 0.9% NaCl solution in 3 mM Tris-HCl (pH 7.4) and again centrifuged at 14000 rpm for 1 min. The pellet was solubilized in 500 \(\mu l\) of a 0.9% NaCl and 0.2% Triton X-100 solution. 100 \(\mu l\) of cell suspension were added to 200 \(\mu l\) of Attophos reagent, mixed for 15 min, after which 100 \(\mu l\) were loaded into each well of a 96-well plate and fluorescence was read at 430 nm excitation and 595 nm emission.
Chapter II. Materials & Methods

3.7. Immunocytochemistry

Immunocytochemistry for expression of osteopontin was performed at the end of the experiment, on 14-day samples (control microparticles and microparticles cultured with cells), as a endpoint assay. The samples were washed twice with 2 ml of PBS 1x (Phosphate Buffered Saline Tablets, Sigma, St. Louis, USA) for 10 minutes. The samples were fixated with 0,5% Glutaraldehyde in PBS 1x (Glutaraldehyde Grade II 25% aqs., Sigma-Aldrich co., St. Louis, USA) and incubated for 30 minutes at 37°C. After fixation, samples were washed again twice with PBS for 10 minutes.

Afterwards cells were permeabilized through the incubation with a 0,2% solution Triton X-100 in PBS, 2 min at room temperature, followed by a washing step, 3 times with PBS for 5 minutes. The primary antibody used was the mouse monoclonal antibody MPIIIIB10 (DSHB, University of Iowa, IA, USA) that was raised against a rat bone extract fraction.

Samples were incubated with the primary antibody in a humidified atmosphere at room temperature with a 1/100 dilution (in a 3% BSA/PBS solution) of the MPIIIIB10 anti-Osteopontin antibody for 1 hour. This incubation period was followed by washing steps (3x) with PBS for 5 min each. For detection of the primary antibody, a 1/100 dilution of the secondary antibody conjugated with a fluorochrome (Alexa Fluor™ 488 goat anti-mouse IgG, Molecular Probes, Leiden, The Netherlands) was added to each lamella, for 1 hour at room temperature and protected from light. After the secondary anti-body incubation the samples were again washed with PBS (3x, 5 minutes each).

The ProLong® Antifade Kit montage medium (ProLong® Antifade Kit (P-7481), Molecular Probes, Leiden, The Netherlands) was prepared and dropped onto the samples and dried for one hour, after which the samples were observed on a fluorescence microscope with a coupled camera/image acquisition system.
3.8. RNA extraction

Total RNA was extracted using the TRIzol reagent (Life Technologies, Gaithersburg, MD). In order to isolate the RNA, chloroform was added to the samples (microparticles with adherent cells), followed by precipitation with isopropyl alcohol.

Samples were transferred to a 1.5 ml tube, centrifuged at 14000 rpm for 2 min and the supernatant was removed. Then, 1ml of TRIzol was added to the pellet, and samples were resuspended about 10 times to lyse the cells. Afterwards, 250 µl of chloroform were added to the samples and the mixture was vortexed. The mixture was then centrifuged at 14000 rpm for 10 min, at 4°C. The top aqueous fraction was collected into a RNase free tube, to which 2 volumes of isopropanol were added. The solutions were mixed and again centrifuged at 14000 rpm, for 30 min at 4°C. The supernatant was discarded and 1 ml of ethanol was added to wash the pellet by centrifugation at 14000 rpm, 6 min, at 4°C. The supernatant was discarded and the samples were air dried for approximately 10 min. The RNA samples were then resuspended in 40 µl of mili-Q sterile water. The concentration and purity of the RNA were measured in a UV spectrophotometer at 260 nm and by calculating the A260/A280 ratio, respectively. The integrity of the RNA was assayed by electrophoresis of the samples in a 1% agarose gel.

3.9. RT-PCR analysis of osteoblastic markers

RT-PCR was performed using a one step procedure. The method was optimized to an amount of 200 ng of RNA. The PCR reaction components were added in the following order: water (ddH2O), Reaction Mix (Invitrogen, Carlsbad, CA, USA), primers, sample RNA and the enzyme, Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The samples were then run in a program with the parameters shown in table III.
Table III. RT-PCR detailed program.

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
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<td></td>
</tr>
<tr>
<td>4 minutes</td>
<td>94°C</td>
<td></td>
</tr>
<tr>
<td>1 minute</td>
<td>94°C</td>
<td>Amplification step</td>
</tr>
<tr>
<td>1 minute</td>
<td>55°C</td>
<td>Repeated 34 times</td>
</tr>
<tr>
<td>1 minute</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>10 minutes</td>
<td>72°C</td>
<td></td>
</tr>
</tbody>
</table>

GADPH, a housekeeping gene, was run as the control.

After completion of the PCR, the products were electrophoresed in a 1.5% agarose gel, together with a 100 bp DNA Ladder (Promega, Madison, WI, USA) and visualized in a Kodak UV imager.

3.10. Mineralization assay: Alizarin Red

The Alizarin Red mineralization assay was performed using the method described by Bodine et al., with modifications. Samples were washed with PBS, and subjected to a fixative 10% v/v solution of formaldehyde in PBS for 15 min. After removal of fixative, samples were washed twice with excess water and covered with Alizarin Red (AR) solution, followed by gentle agitation in an orbital shaker for 20 min. The AR solution was then removed and the samples washed four times with mili-Q water. The samples were observed in an optical microscope with a coupled SPOT camera.
3.10.1. Alizarin red staining extraction

The protocol for extraction of the Alizarin Red staining was adapted from Gregory et al.\textsuperscript{21}. 500 µl of acetic acid (10% v/v) were added to the stained samples, incubated for 30 min at room temperature with mild shaking. The monolayer was then scrapped off the plate, transferred to a 1.5 ml tube and vortexed for 30 s. The slurry was overlaid with 300 µl of mineral oil to prevent evaporation and heated at 80ºC for 10 min. Then the samples were centrifuged for 15 min at 14000 rpm. 300 µl of the supernatant was transferred to a 1.5 mL tube and mixed with 100 µL of a 10% (v/v) solution of ammonium hydroxide. 100 µL aliquots were transferred to a 96-well plate and the absorbance read at 430 nm. At least 3 independent experiments were performed, each in triplicate.

4. INCORPORATION AND RELEASE OF BIOLOGICALLY ACTIVE MOLECULES USING STARCH-BASED MICROPARTICLES

The developed starch-based microparticles aimed to be used as drug delivery systems. To evaluate their capability to serve as such carriers, two groups of biologically active agents were selected to be incorporated into the matrix of starch-based microparticles: corticosteroids and growth factors. Each groups' representative molecule was incorporated into starch-based microparticles and the following parameters were evaluated: loading efficiency, release profile and maintenance of the biological activity of the molecule after incorporation and release.
4.1. Incorporation of biologically active molecules into starch-based microparticles

A blend of starch and polylactic acid, SPLA (50% cornstarch-50% PLA, by weight) was used as the raw material for the production of the microparticles using the modified solvent evaporation emulsion method described in section 1.3.1. This material and this method were chosen for the incorporation of biologically active molecules because it combined the shortest time of production and the particles with longer degradation times and higher stability, which could influence the activity of the molecule to be incorporated.

Dexamethasone ≥ 98% (HPLC grade, Sigma, St Louis, MO, USA), 16α-Methylprednisolone ≥ 99% and 16α-Methylprednisolone acetate ≥ 99.3% (both in house produced) were used as the biologically active molecules for entrapment and release studies. All other chemicals used were reagent grade (Panreac, Barcelona, Spain) except methanol that was HPLC grade (Riedel-de-Haën, Sigma/Aldrich, Seetze, Germany).

Values of 2.5%, 5% and 10% wt/wt (dexamethasone/polymer) were tested in order to determine the ratio of agent to polymer that achieved the highest encapsulation efficiency. Based on these results, 10% was chosen as the ratio to be used further.

For the synthesis of microparticles, the steroid (10% weight of polymer) was mixed with SPLA and partially dissolved in methylene chloride very briefly. The microparticles were then washed and separated from the reaction solution by filtration. The reaction solution was stored for quantification of the non-loaded corticosteroid. Steroid-loaded microparticles were freeze-dried and stored in a desiccator until further use. This procedure was performed separately for each of the corticosteroids used, namely dexamethasone (DEX), 16α-Methylprednisolone (MP) and 16α-Methylprednisolone acetate (MPA). At least three independent experiments were performed for each of the corticosteroids, being the results reproducible.

This same method was followed for the incorporation of PDGF. Microparticles containing Platelet-Derived Growth Factor (Human recombinant Platelet-Derived Growth Factor-BB (hPDGF-BB), R&D Systems, Minneapolis, USA) were produced by co-encapsulating PDGF with bovine
serum albumin (BSA) (fraction V, Fisher, USA), using the methodology described above. BSA is commonly used as a carrier[22] for the encapsulation and protection of biologically active molecules from the harsh organic solvents, which can cause inactivation of the molecules. BSA and PDGF were mixed prior to addition to the SPLA in methylene chloride solution at a ratio of 1:20 000 of PDGF:BSA (i.e. 10 µg PDGF in 200 mg BSA). The resultant microparticles were sieved into 3 different size ranges: <40 µm, 40-210 µm and >210 µm, and particles with size >210 µm were chosen for this study. Particles with size >210 µm were chosen for this study, following studies in which this same size range was used for the culture of osteoblastic cells[23]. Microparticles incorporating BSA alone were also produced, as a control. Throughout this study the following controls were used: (i) SPLA microparticles (GF-free) and (ii) BSA-loaded microparticles.

4.2. Loading efficiency

The loading efficiency for the different steroids was determined by measuring the amount of corticosteroid remaining in the reaction medium (unloaded) where the microparticles were produced. Aliquots of 1 ml of the reaction medium were taken and the amount of DEX, MP and MPA present in each sample measured by High Performance Liquid Chromatography (HPLC) as described below. The results were obtained from three independent experiments run in duplicate with reproducible results and the mean value is reported.

The loading efficiency was calculated using equation 3.

\[
\text{Loading efficiency} \% = \frac{(\text{CS}_i - \text{CS}_r)}{\text{CS}_i} \times 100\%
\]

(equation 3)

where CS\(_i\) is the initial amount of corticosteroid to be incorporated and CS\(_r\) the amount of corticosteroid remaining in the reaction medium (unloaded), where microparticles were synthesized.
Loading efficiency for PDGF-SPLA microparticles was determined differently from the described for corticosteroids. The basis for this was that the amount of PDGF (0.0000001% wt) to be incorporated was considerably lower than that of dexamethasone (10% wt), therefore the non-incorporated growth factor remaining in the reaction medium (PVA solution, 200ml) would be very diluted and might lead to erroneous quantification. The loading efficiency was defined as the ratio of PDGF encapsulated in the SPLA microparticles to the initial amount of PDGF, as in equation 4.

\[
\text{Loading efficiency PDGF (\%) = \frac{\text{PDGF incorporated}}{\text{PDGF initial}} \times 100\%}
\] (equation 4)

To this end, 20 mg of PDGF- SPLA microparticles were dissolved overnight in 1 N NaOH and PDGF was measured by an Enzyme-Linked ImmunoSorbent Assay (ELISA) specific for human PDGF-BB (R&D Systems, MN, USA). BSA loading efficiency from BSA-SPLA microparticles was measured by the Bradford method.

4.3. *In vitro* release

*In vitro* release studies were conducted in phosphate buffer saline solution (PBS), 0.1M, pH 7.4. Pre-weighted corticosteroid-SPLA microparticles were immersed in sterile PBS at a ratio of 5 mg/ml under mild agitating conditions, at 37°C. At pre-determined time points, PBS aliquots were sampled for further quantification of the released corticosteroid. The results were obtained from two independent experiments run in duplicate with reproducible results and the mean value is reported.

Release studies for PDGF-SPLA microparticles were conducted with some modifications. Since the amount of PDGF to be incorporated was very reduced and having into consideration the loading efficiency results, the amount of microparticles in the release studies was increased to 200 mg per 5 ml of PBS-AB (Phosphate-buffered solution containing 1% of antibiotics – Pen-Strep).
The use of PBS with supplemented antibiotics was used to prevent any contamination of the samples.

Controls for the study included PBS-AB, SPLA microparticles (GF-free) and BSA-SPLA microparticles (table IV), subjected to the same conditions as described for PDGF-SPLA microparticles.

Table IV. Controls and samples used in the release experiments.

<table>
<thead>
<tr>
<th>Control/Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-AB</td>
</tr>
<tr>
<td>SPLA microparticles (GF free)</td>
</tr>
<tr>
<td>BSA-SPLA microparticles</td>
</tr>
<tr>
<td>PDGF-SPLA microparticles &gt;210 µm</td>
</tr>
</tbody>
</table>

4.4. Quantification of the released molecules

For the quantification of released corticosteroids, samples were extracted 3 times with a mixture of hexane and ethyl acetate (1:1), to selectively remove the corticosteroids. The extracts were combined and evaporated under a nitrogen flow. The residues were re-dissolved in methanol and subjected to analysis by HPLC.

The HPLC system consisted of an auto-injector Midas Injector Spark, and a Beckman System Gold Solvent Module 126 coupled to a Detector Module 166. The separation of the samples was performed in a PurospherStar RP-18e column (250x4.5 mm, 5µm particle size; Merck, Germany), using methanol and water as solvents. The following gradient was utilized: 50% methanol for 10 min, 30% methanol-70% water for 25 min. The flow rate was 1 ml/min and the detection wavelength used was 254 nm. Quantification of DEX, MP and MPA was performed using the internal standard method using Triamcinolone (Lederle, New York, USA) as the reference compound.
The released PDGF was quantified using an ELISA kit, by comparison to a standard PDGF-BB curve, according to the manufacturers' instructions. All experiments were performed at least in duplicate.

4.5. Evaluation of the maintenance of the biological activity of the released molecules

When a molecule is incorporated inside a release system, it is vital that its biological activity is maintained throughout loading, incorporation and release. For dexamethasone and the other corticosteroids the maintenance of this activity was assessed by the spectra obtained by HPLC (used to quantify released corticosteroids) by comparison of the specific peaks and retention time with the controls.

To evaluate if PDGF incorporated into SPLA microparticles is still active after incorporation and release, it was used a culture of MC3T3-E1 cells.

However, before analyzing the biological activity of released PDGF it was necessary to determine the minimal concentration of PDGF producing a mitogenic effect over MC3T3-E1 cells. Several concentrations of exogenous PDGF - that ranged from 0 to 50 ng/mL – were tested beforehand.

Cells were seeded in 12-well tissue culture plates (Corning, USA) at a density of 12600 cells/cm². 24 hours after cell seeding, the culture medium was removed and replaced by fresh DMEM medium supplemented with the following concentrations of exogenous PDGF: 0, 2, 4, 10, 20 and 50 ng/mL. After 72 h, DNA levels were measured by the PicoGreen dsDNA quantification kit, as described in section 3.5. The results were obtained from three experiments.

After determining the minimal concentration of PDGF necessary to produce a mitogenic effect for MC3T3-E1 cells, the same cell density and experimental setting was used to test the samples and controls after 3 and 6 days, as summarized in table V.
Table V. Controls and test conditions used for the evaluation of the bioactivity of released PDGF.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Content of culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>DMEM</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + PBS-AB</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + supernatant of SPLA microparticles (GF free)</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + supernatant of BSA released from microparticles</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + exogenous BSA</td>
</tr>
<tr>
<td>Positive control</td>
<td>DMEM + BSA-SPLA microparticles</td>
</tr>
<tr>
<td>Sample</td>
<td>DMEM + supernatant of PDGF released from microparticles</td>
</tr>
<tr>
<td>Sample</td>
<td>DMEM + PDGF-SPLA microparticles</td>
</tr>
</tbody>
</table>

5. Statistical analysis

Results are expressed as mean ± standard deviation.

Differences between experimental results were analyzed by the Student t-test, with the threshold for statistical significance set at p<0.01 or p<0.05, according to the different studies.
References


Chapter III

Microparticulate systems based on natural origin materials

1. PARTICULATE SYSTEMS: FUNDAMENTALS

It has been a long path for particulate systems in biomedical applications. Since long time ago that these micron and nano sized systems synthesized from the most varied materials find application in the biomaterials field, mainly as drug delivery carrier systems. The aim of drug delivery systems is to facilitate the dosage and duration of the drug effect, causing the minimal harm to the patient and improving human health. Typically, they allow for the reduction of the dosage frequency and are non-toxic. Back in time, these systems designed for the controlled delivery of drugs were found extremely promising for several applications, such as the delivery of insulin, contraceptives, cancer therapeutics among others.

Polymer microparticles have attracted attention as carrier matrices in a wide variety of medical and biological applications, such as affinity chromatography, immobilization technologies, drug delivery systems and cell culturing.

* This chapter is based on the following publication:

Various parameters including particle size and size distribution, porosity and pore structure and surface area are considered to describe the overall performance of polymeric microspheres in these applications\textsuperscript{32}.

At the present, the applications are diverse, ranging from drug delivery devices – where these particulate systems find their primary field of action – to other biomedical applications, namely those related with detection and analysis applications\textsuperscript{20, 33-35}. These systems could also be used in the Tissue Engineering field, has it has been reported by several authors\textsuperscript{36-42}.

1.1. Natural origin materials

The development of particulate systems is in constant evolution, and every year more and more new materials are used to synthesize them. PLA, PGA and their co-polymers have been widely used, but it is well known the problems associated with these materials\textsuperscript{43-47}. Many other synthetic polymers have been studied. These polymers have found several applications, but are clearly not adequate for others.

This led the scientific community to turn to natural origin materials, as an alternative group of materials. The main characteristics of useful natural origin materials are their availability/renewability, low cost, biodegradable character, easy of processing, among others. These materials can be processed alone or in combination with other polymers or with other groups of materials (ceramics, metals).

Polymers from crab shells, such as chitin and its deacetylated derivative, chitosan, proteins from plant origin, such as soybean protein, starch from corn, potato, cassava, casein, albumin, collagen, gelatin among others are currently being studied within the biomaterials field.

Proteins are a diverse group of biopolymers widely studied for biomedical applications. Albumin, collagen and gelatin are three of the most studied proteins to form
microcapsules/spheres. Their main disadvantages are their susceptibility of denaturation in adverse environments, the risk of allergy, and potential disease transmission.

Chitin and its derivative chitosan are now being widely used for several drug delivery applications. Chitin is, after cellulose, the most abundant polymer found in nature. Chitin can be complexed with iron to form microspheres that can be used as possible drug delivery systems targeted for liver, due to their bioavailability.

Chitosan \([\beta(1-4)2\text{-amino-2-deoxy-D-glucose}]\) is a cationic polysaccharide derived by hydrolysing the aminoacetyl groups of chitin, hydrophilic, biocompatible and biodegradable polymer. Chitosan has been used for several applications, namely as delivery systems for anticancer drugs, such as doxorubicin, 5-fluorouracil, nucleic acids carriers, such as DNA-chitosan nanoparticle complexes. This material can also be modified to accommodate other bioactive agents such as in chitosan succinate and hydroxamated chitosan succinate (both crosslinked with iron) that were found successful in the encapsulation and prolonged release of theophylline.

Works on soy and casein proteins has revealed the potential of these materials for biomedical applications. Soy thermoplastics have been developed so as to constitute dual release systems when processed by a double-layer co-injection molding method, as well as their ability to incorporate and release theophylline in a two stage process. When processed as transdermal delivery systems, such as membranes, these were found cytocompatible when tested in vitro.

Also starch has been widely used, usually modified, either by blending with other polymers or by chemical modification of its structure. Several works performed with starch, namely in the microspheres form have shown to be nasally inhaled to deliver proteins and drugs, as well as its processing as porous scaffolds, that could be combined with other materials to yield materials with adequate mechanical, chemical and biocompatible character.

Other materials have been used, but their description would become this chapter too extensive.
1.2 Blends and composite materials

The development of polymeric matrix composite materials aims to combine the most desired properties of two or more materials\textsuperscript{36}, either between the same group of materials (polymers) or between different classes of materials (polymer-ceramic). Composite materials aim to combine the best properties of each material. Depending on the application, one should choose the materials that may bring together the desired properties, and to tailor them for the foreseen application.

In the polymer field, there are frequently examples of the combination of two different polymers to yield a final material that has enhanced properties compared to the ones displayed by each of the individual materials. Examples are found in the literature, such as the combination of chitosan with xanthan\textsuperscript{69, 70}, two polysaccharides that when complexed in the form of microspheres presented a slower degradation profile when compared with chitosan microspheres alone. Hyaluronic acid was also blended with chitosan to create microspheres with mucoadhesive properties\textsuperscript{50}, that can be very useful in intranasally administrated drugs. Also natural and synthetic polymers can be combined, such as chitin and PLGA, that where blended to form biodegradable microspheres for protein delivery\textsuperscript{25}.

Chitosan can also be combined with ceramic materials, such as coralline hydroxyapatite\textsuperscript{71}, that were found to be effective in the entrapment and zero-order release kinetics of gentamicin, thus presenting a great potential for bone and dental applications.

Hydroxylapatite is a ceramic material widely used for creating composite polymer-ceramic materials. For instance, embedding hydroxyapatite particles into reconstituted fibrous collagen was found capable of promoting osteoblast attachment and growth, thus rendering it suitable for clinical applications\textsuperscript{72}.

Other combination of polymers with ceramic materials, namely starch-based materials and Bioactive Glass 45S5, has shown that microparticles with spherical shape were formed, entrapping Bioactive Glass 45S5\textsuperscript{36}. 
2. APPLICATIONS OF PARTICULATE SYSTEMS IN TISSUE ENGINEERING

Particulate systems find their application in the biomedical field mainly as drug delivery vehicles. In the field of Tissue Engineering, particulate systems are not used per se, but usually produced and then molded into porous scaffolds. This can be a strategy to combine scaffolding and drug delivery, but work still has to be done in order to optimize properties such as pore dimension or mechanical properties namely for bone related applications. The advantages of these systems for Tissue Engineering applications comes from their ability to be targeted to specific sites of action, and their biodegradability, that will allow them to disappear when they are no longer required. These properties can be advantageous when applications like drug or cell delivery are foreseen, where drugs or cells will be released in particular time points. For instance, a dual release system that may entrap cells and its differentiation agents, cells that could only be released as the material degrades, and during that time the differentiation agents could act in the cells, could be created.

The fast development of Nanotechnology has pushed forward the interest in the small-scale materials. One can easily predict that the Nano era, that is just now beginning, will allow for enormous developments in the applications of these micron and submicron size systems.

2.1. Bone and cartilage

Concerning hard tissues such as bone, not many advances have been made in the use of particulate systems in tissue engineering. Ceramic materials are sometimes used in the form of particulate materials for filling of small defects, especially in maxillofacial and dental applications. As for polymers, they are so far restricted to be used as porous scaffolds, either by polymer processing or “double-processing”, where particles are produced and then sintered to form 3-D scaffolds.
Thus, a strategy that would combine 3-D-porous scaffolds with micron size particle systems acting as drug delivery/cell encapsulation systems creating the perfect scaffold, could constitute a leap forward in bone and cartilage tissue engineering.

Bearing this in mind, starch-based materials present themselves as a good alternative, since they can be processed into porous scaffolds with interesting properties\textsuperscript{62, 63, 77-81}, and as it will be described, into particulate systems that can be used as delivery systems or as composite materials (with ceramics) for reinforcement.

3. STARCH-BASED MATERIALS

Reis and co-workers\textsuperscript{61, 82-91} have previously demonstrated the potential starch-based materials for several biomedical applications. These materials have been proving their potential for biomedical applications such as bone cements\textsuperscript{86}, drug delivery systems\textsuperscript{85, 92}, bone tissue engineering scaffolds\textsuperscript{83, 84}, among other possible applications.

Their adequate profile can be evidenced by their easiness of processing, their ability to be combined with other polymers or ceramic materials (such as hydroxylapatite or bioactive glasses), their renewable and natural origin nature, their enzymatic degradation\textsuperscript{93}, water uptake ability\textsuperscript{94, 95}, their physical and chemical properties that can be tailored and modified according to the foreseen application\textsuperscript{62, 96-98} as well as its unusual (for a biodegradable material) good biological performance\textsuperscript{91, 99}.

Different blends of starch with other polymers, such as polylactic acid, polycaprolactone, ethylene vinyl alcohol and cellulose acetate have been studied in order to obtain materials that can have a combination of suitable properties, can be easily processed and are biocompatible\textsuperscript{88, 91, 100, 101}. 

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3.1. SPLA and composite particulate systems

In this work, a blend of cornstarch and polylactic acid (PLA) was used to synthesize particles that can be applied as drug delivery carriers, both alone and in combination with scaffolds. In the latest case the aim is to achieve a release profile closer to the desired, since release from three-dimensional porous scaffolds occurs too fast in the majority of the cases. Thus, by combining micron size delivery systems and scaffolds, it would be possible to tailor the release profile of growth factors or other bioactive agents that should act in the implantation site.

A simple method were the blend of starch and polylactic acid was dissolved in an organic solvent and then emulsified with a stirring aqueous solution was used to prepare the particles. This method yielded particles of a size of less than 10\(\mu\)m up to 350 \(\mu\)m (figure 1.), with a spherical morphology, whose surface alternates between smooth areas (attributed to PLA) and porous areas (starch phase).

![Size distribution of the developed SPLA particles as determined by sieving. The size distribution shows that the particle size between 210-350 \(\mu\)m predominates over all other sizes.](image)

**Figure 1.** Size distribution of the developed SPLA particles as determined by sieving. The size distribution shows that the particle size between 210-350 \(\mu\)m predominates over all other sizes.
Figure 2. Scanning electron microscopy image representative for SPLA and SPLA/BG particles. No differences were found between polymer and composite particles. Regarding the morphology, smooth areas and porous areas are observed in the particles.

Particles composed of SPLA and 30% (w/w) of Bioactive Glass 45S5 (BG 45S5) were also synthesized, by mixing BG 45S5 with SPLA and then forming the particles as described for SPLA. No differences on both size distribution and morphology (figures 1. and 2.) could be found as compared to polymeric SPLA particles.

However, when evaluating their water uptake ability and degradation profile (through weight loss and reducing sugars quantification) it was found that the composite particles degrade slower than the polymeric ones (figure 3.).

Figure 3. Water uptake (A) and weight loss (B) profiles for SPLA and SPLA/BG particles. Composite particles have a similar water uptake profile as SPLA particles, differing in the weight loss, which is lower than the one observed for the polymeric particles.
It was also found that the composite materials are bioactive upon immersion in a solution simulating the human blood ionic composition, as seen by the formation of a calcium-phosphate layer at the surface of the particles\textsuperscript{36}.

SPLA particles have already been studied for their ability to encapsulate and release bioactive agents, being proved their potential to be used as drug delivery carriers\textsuperscript{28}, namely of growth factors or other bioactive agents.

### 3.2. Soluble potato starch and composite particulate systems

Paselli 2 starch is a potato starch chemically modified by ATO (Wagningen, The Netherlands) as to become soluble in water. This material was used with an emulsion crosslinking method based on the creation of a water-in-oil emulsion. Starch is crosslinked with trisodium trimetaphosphate (TSTP). Briefly, this method can be described as the dissolution of starch and TSTP in water, which is then emulsified with paraffin and a surfactant for emulsion formation. When the reaction is completed, the particles are extensively washed with water and ethanol and then freeze-dried. Due to particle aggregation, no size separation was performed for this material, but sizes up to 1 mm were observed, in particular for composite Pa2/BG 45S5 particles, synthesized as described for polymer particles, by mixing starch and BG 45S5 (30% w/w) in the initial step of the method.

In figures 4 and 5, representative images of Pa2 (figures 4A, 4B) and of Pa2/BG 45S5 (figure 5A, 5B) particles are shown.
Chapter III: Microparticulate systems based on natural origin materials

Figure 4. Scanning electron microscopy images representative of Pa2 particles (A, B). Regarding the size distribution, particle size ranges from few microns (A) to hundreds of microns (B). Morphological evaluation shows that the particles seem to be composed of smaller particles, all brought together. This feature creates pores in the particles, but they do possess a dense matrix with only very small pore sizes.

The size range of these particles goes from a few microns (fig. 4A) to hundreds of microns (fig. 4B). The polymer particles present a spherical surface, which seems to be composed from several smaller particles, thus rendering this material porous, but with a dense matrix were only some very small pores observed (data not shown).

When analyzing the composite materials (fig. 5A, 5B), the size range is similar to the polymeric particles. However, when comparing the morphology, it is clearly different from the polymeric ones.

Figure 5. Scanning electron microscopy images representative of Pa2-BG composite particles (A, B). Size range is the same as compared with polymeric particles, however differences in the morphology do occur. Composite particles are more regularly spherical, with a smoother surface.
In Pa-BG particles the cross-section of a composite particle cut to expose its interior shows the BG 45S5 granules dispersed in the starch network.

Composite Pa-II-BG particles present a smoother surface when compared to Pa2 particles. The presence of BG45S5 in the particles is shown dispersed in the particles matrix, as seen in figure 5B (BG 45S5 granules pointed by arrows).

The bioactivity of both Pa2 polymeric and Pa2-BG composite particles is being evaluated. Regarding their water uptake (figure 6A) and weight loss (figure 6B) profiles, as for water uptake the values are higher for composite particles. This feature can be explained by high water uptake at the interaction between polymer and ceramic phases. Pa2 polymer particles have a higher loss of weight when immersed in a phosphate buffered saline solution, proved by the higher amount of quantified reducing sugars, that measure degradation of small weight starch chains.

**Figure 6.** Water uptake (A) and weight loss (B) profiles for Pa2 and Pa2-BG particles. Composite particles have a slight higher water uptake profile as Pa2 particles, differing in the weight loss, which is lower than the one of polymeric particles.
3.3. Other starch-based particle systems: SCA, SEVA-C

Other starch-based materials were used for producing micron size particles. For example, a blend of starch with cellulose acetate (SCA) was used for the synthesis of particles. The method chosen was as described for Paselli II (soluble starch) particles. The morphology was evaluated prior to any other characterization and it shows (figure 7A) a spherical morphology of the particles, with an average size of 160 µm, and with a surface alternating between smoother areas and rougher areas (figure 7B), with a dense matrix.

![Figure 7. Scanning electron microscopy images of a representative SCA particle (A) and detail of SCA particles’ surface (B).](image)

When evaluating the ability of SEVA-C, a blend of starch and ethylene vinyl alcohol to be processed as particulate systems, using the method described above for Paselli II, this material has shown to form microparticles with spherical shape (figure 8A), with sizes up to 700 µm. These particles present a rough surface, with some porosity (figure 8B).
4. CONCLUSION

Natural origin materials are in the biomedical field to stay. Their potential for biomedical applications has been coming to scene, and scientists are realizing their potential.

From crab shells to crop reserves, from plant proteins to coralline materials, from animal to plant by-products, there is a vast panorama from where to choose, and many more will be continuously brought front as the search for the perfect material continues. Combining expertise from different fields such as chemistry, materials science, biology and other sciences can lead the scientific community to develop adequate materials.

For some biomedical applications, natural origin materials cannot alone suffice the needs. However, their combination with other materials, either of natural or synthetic origin can create new materials that can in fact combine the best properties from each material, pushing development forward.

In this context, starch-based polymers are one of the good alternatives, either alone or in combination with other materials – for instance, ceramic materials such as hydroxylapatite or bioactive glasses. The ability of starch-based materials to be tailored, modified, as well as their
biological performance and variety of ways of processing them, has brought up their potential to be used as scaffolds, drug carrier systems, bone cements, among others.

It is our firm belief that the future lies in natural origin materials, and that by learning with Nature we can actually create materials that can perform what they were designed to: improve, regenerate, heal, ultimately, give hope for a better quality of life of patients worldwide.
REFERENCES

Chapter III: Microparticulate systems based on natural origin materials


51. Mao H., 2001, Chitosan


Chapter III: Microparticulate systems based on natural origin materials


Abstract

The aim of the development of composite materials is to combine the most desired properties of two or more materials. In this work, the biodegradable character, good controlled release properties and natural origin of starch-based biomaterials are combined with the bioactive and bone-bonding properties of bioactive glass (BG).

Novel, bioactive composite starch-BG microparticles were synthesized starting from a blend of starch and polylactic acid (SPLA, 50%:50% wt) with BG 45S5 power using a simple emulsion method. Morphological and chemical characterization showed that these particles exhibited a spherical morphology with sizes up to 350 µm and that BG 45S5 was incorporated successfully into the composite particles. Upon immersion in a solution simulating body fluids, for periods up to three weeks, their bioactive nature was confirmed, as a calcium-phosphate layer, resembling biological apatite was formed onto their surface.

* This chapter is based on the following publication:
The short-term cytotoxicity of these materials was also tested by placing 24h leachables of the materials-extracted in culture medium in contact with a fibroblastic cell line (L929) up to 72 hours. At this time period, two biochemical tests – MTT and total protein quantification – were performed. The results showed that these materials are not cytotoxic.

These results constitute the basis of future encapsulation studies using bone-acting therapeutic agents such as Bone Morphogenetic Proteins or other bone relevant factors. The particles developed here may be very useful for applications in which controlled release, degradability and bone-bonding ability are the main requirements.

1. INTRODUCTION

A bioactive bone-bonding material is one that elicits a specific biological response at the interface of the material, which results in the formation of a bond between the tissues and the material\textsuperscript{1,2}. Up to date, no polymer displayed by itself a bioactive behavior (unless pre-treatments have been performed). One strategy to promote their bioactivity is to combine polymers and ceramics, thus creating composite materials that exhibit a range of properties that derive from both constituent materials. This way, it is possible to create materials that mitigate the lack of satisfactory properties of both polymers and ceramics\textsuperscript{2}. The use of starch-based materials can be justified by the well-known acidification phenomenon when using systems constituted by polylactic acid. Using natural origin materials this problem can be readily overcome since starch can be degraded within the body by several enzymes\textsuperscript{3}, resulting in degradation products (carbohydrates) that can be readily metabolized and excreted.

Starch-based polymers are being studied for a wide range of bone related therapies applications, ranging from tissue engineering scaffolds\textsuperscript{4}, to bone cements\textsuperscript{5} and drug delivery systems\textsuperscript{6}. Its natural origin, together with its mechanical properties\textsuperscript{7-9} and biocompatibility\textsuperscript{10,11}
support the potential of starch-based materials in the biomedical field. As for Bioactive Glass 45S5 (with a nominal composition of 46.1% SiO$_2$, 24.4% Na$_2$O, 26.9% CaO, 2.6% P$_2$O$_5$ mole%), its rapid rate of surface reaction leads to fast tissue bonding when compared to other ceramic materials. Biological properties of Bioactive Glass 45S5 have been extensively studied$^{12-14}$ and, as shown in vitro, it was found to enhance extracellular matrix formation and promote the osteoblastic phenotypic expression$^{15}$.

Starch microparticles have already been shown to be excellent for the controlled release of meclofenamic acid, an anti-inflammatory agent, and for the release of glucocorticoid agents such as dexamethasone$^{6,16}$. When combining these properties with the advantageous bone-bonding properties of Bioactive Glass 45S5, there is a distinct potential for these particles to be used as controlled release systems of either bone-acting drugs or growth factors. In principle, these systems would be able to bond to bone and at the same time act as drug release systems. In theory, the presence of a bone bonding material (Bioactive Glass) would enhance small defect bone repair while, simultaneously, the biodegradable material would act as a scaffold for cell growth, by releasing incorporated growth factors. This release would stimulate cell proliferation and differentiation, thus achieving a faster repair.

In the present study we synthesized composite microparticles composed by a starch-based material and by Bioactive Glass 45S5. These particles display a bioactive behavior, as seen by the formation of a calcium-phosphate layer resembling biological apatite at their surface upon immersion in a solution simulating body fluid. As for all newly proposed biomaterials, a cytotoxic screening must be performed as a preliminary step in the process of the determination of the compatibility with biological systems (biocompatibility). In this work the short-term cytotoxicity of the developed materials was tested by means of MEM extraction tests.
2. MATERIALS AND METHODS

2.1. Materials

A commercial blend of starch and polylactic acid (SPLA, 50%:50% wt) (Novamont, Italy) was used as raw material for the production of the microparticles. Bioactive Glass 45S5 (46.1% SiO$_2$, 24.4% Na$_2$O, 26.9% CaO, 2.6% P$_2$O$_5$, mole%) was obtained from MO-SCI (Rolla, MO, USA) and ground before use to a particle size smaller than 60 µm.

For particle synthesis, methylene chloride and polyvinyl alcohol 87-89% hydrolyzed, used as received. The following chemicals were used for preparing the simulated body solution, herein designated as Tris buffer with electrolytes: 0.05 M Tris buffer solution, Trizma-HCl, sodium chloride (NaCl), potassium chloride (KCl), sodium hydrogen carbonate (NaHCO3), magnesium chloride hexahydrated (MgCl2.6H2O), magnesium sulphate heptahydrated (MgSO4.7H2O), dipotassium hydrogen phosphate anhydrous (K2HPO4), calcium chloride anhydrous (CaCl2), used as received.

2.2. Synthesis of SPLA and SPLA/BG particles

SPLA particles were produced by an emulsification method adapted from Qiu et al. The method was modified as follows: 1 g of SPLA was dissolved in 10 ml of methylene chloride and this solution was added to 200 ml of a stirring 0.5% Polyvinyl alcohol (PVA) solution and emulsified for 5 hours in a Caframo dual-range stirrer (Fisher Scientific, USA) at a speed of 660 rpm/min. The particles were then washed and separated from the solution by filtration. They were dried, separated by size and stored in a desiccator.

The SPLA/BG composite particles were produced following the methodology described above, to which 30% of the starch-based material weight of BG 45S5 was added to the starch solution.
2.3. SPLA and SPLA/BG particles: morphological characterization

The synthesized particles were morphologically characterized by light microscopy, scanning electron microscopy (SEM) and size distribution was determined for each condition by sieving. The particles were observed and photographed in an optical microscope with an image acquisition system (high resolution color video camera and Image-Pro Plus analysis software, Media Cybernetics, Silver Spring, Maryland, USA). SEM analysis was performed on gold-coated samples.

Size distribution was obtained by determining the weight of each of the obtained fractions after sieving in a sonic sifter (ATM sonic sifter, model L3P, ATM Co., Milwaukee, USA). For the determination of the size distribution measurements were made at least in triplicate.

2.4. Immersion - bioactivity evaluation

SPLA and SPLA/BG particles of a size range between 210-350 μm were used for in-vitro testing of their bioactivity in a 0.05 M tris hydroxymethyl aminomethane-buffered solution complemented with electrolytes typical for plasma. The use of this solution was described previously\textsuperscript{18-20}. SPLA-based particles were immersed in the Tris buffer with electrolytes’ solution at a ratio of 5 mg/ml and 3 mg/ml, for SPLA and SPLA/BG particles, respectively, in a CO\textsubscript{2} incubator at 37°C. The vials were placed on an orbital shaker at a rotational speed of 150 rpm for up to 21 days without solution exchange. At designed time points (1, 2, 3, 7, 10, 14 and 21 days), the samples were removed from the shaker, the pH was measured and the particles separated from the solution by filtration. The later were then dried and stored in a desiccator until further analysis. The post immersion solutions were used to perform chemical analysis, namely for changes in Ca, Si and P concentrations. Ca and Si concentrations were measured by Atomic Absorption
Spectrophotometry. For the Ca measurements a solution of 1% LaCl₃ was used, while the Si measurements were performed directly from the post immersion solution. For the P measurements a UV-Visible spectrophotometer was used at 400 nm and a colorimetric method was employed. Samples were also analyzed by ICP-OES (Inductively Coupled Plasma- Optical Emission Spectroscopy).

Further characterization of the immersed particles was performed using Fourier Transform InfraRed Attenuated reflection (FTIR-ATR), Scanning Electron Microscopy/Energy Dispersive X-Ray Spectroscopy (SEM/EDS) analysis and X-Ray Diffraction (XRD) analysis.

2.5. Short-term cytotoxicity screening

For cytotoxicity evaluation by indirect contact with the materials, two different tests were performed, namely MTT and total protein quantification test.

The cells used in this study were an immortalized cell line of subcutaneous areolar fibroblasts from mouse origin purchased from Collection of Cell Culture (ECACC, UK, ref. 85011425), designated L929. The cells were grown in monolayer systems in Dulbecco’s Modified Eagle Medium supplemented (as described in the next section) and used between passages 6 and 12.

2.5.1. MTT

The MTT test is a biochemical test widely used to assess cytotoxicity by measuring cell viability and proliferation in a qualitative way. This biochemical test is based in the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide), (which is water-soluble and has a yellow tonality) by the cell mitochondrial enzyme succinate dehydrogenase, yielding a purple color salt insoluble in water. The salt absorbs at a wavelength of 570 nm and since only living cells have the capability of metabolize the MTT, it gives a measurement of the viable cells.
The procedure followed for this test was as described previously and corresponds to the ISO MEM Elution test for short-term cytotoxicity assessment. Briefly, SPLA and SPLA/BG particles were placed in conic tubes with DMEM culture medium and placed under constant shaking (60 rpm) at 37ºC for 24 hours for the materials to release eventual leachables (formation of extracts from the materials). An extraction control was performed, which consists on DMEM culture medium, placed in the same conditions as the samples. This control was performed to assure that the extraction conditions by themselves do not alter the properties of the culture medium. After 24 hours the extracts were filtered through a 0.45 µm pore size filter, placed in contact with an 80-90% confluence cells’ monolayer and further incubated for 72h. After this time period the viability of the cells is evaluated by the MTT test by measuring the optical density. All the materials were tested in 10 replicates for each extract for at least two independent experiments with reproducible results. The results are expressed as a percentage of the control (scored as 100% viability) as mean ± standard errors.

2.5.2. Total Protein Quantification

The method that was used to quantify the total protein uses the Micro BCA Protein Assay Reagent Kit, in which bicinchoninic acid (BCA) is the detection reagent for a Cu complex, which is formed when Cu²⁺ is reduced by proteins in an alkaline environment. The purple color product is due to the chelation of two molecules of BCA with one Cu ion. This complex is water-soluble and absorbs at 562 nm, and its optical density is linearly correlated with protein concentration.

The procedure followed is as described for the MTT test. After the 72 hours incubation, the cells are washed with PBS and the BCA reagent is added. After an incubation period, the optical densities were measured. Total protein (in µg) was determined using a BSA standard curve.

All the materials were tested in 10 replicates for each extract for at least three separate experiments, with reproducible results. The results are expressed as percentage of the control (scored as 100% viability) as mean ± standard errors.
3. RESULTS & DISCUSSION

3.1. Synthesis of SPLA and SPLA/BG particles

The size distribution for SPLA and SPLA/BG particles (Figure 1) clearly shows that the size range of 210-350 µm predominates, while particles with sizes above 350 µm are absent and below 40 µm are only a minor fraction. This allows stating that the addition of BG to form composite particles does not significantly affect the size distribution of the starch-based particles.

Figure 1. SPLA and SPLA/BG particle size distribution. After sieving each size interval was weighted and its relative amount was determined. The graph shows that both conditions present similar size distribution patterns, which indicates that the addition of BG 45S5 powder does not seem to affect the size distribution of the particles.

3.2. Morphology of SPLA and SPLA/BG particles

The morphology of polymer and composite particles was evaluated by optical microscopy (figure 2) coupled to an image acquisition system. It was also analyzed using scanning electron microscopy (figure 3).
Figure 2. Light optical microscopy images of SPLA (A) and SPLA/BG (B) particles. No morphological differences between polymeric and composite particles are visible.

Figure 3. SEM image of SPLA (A, B, a detail of the rougher surface) and SPLA/BG particles (C). There are no clear morphological differences between the two types of particles.
The morphology of the particles as revealed by light microscopy was indifferent of the type of particles. The particles were approximately spherical, which was further confirmed by SEM analysis. In addition, this examination also showed that their surfaces were quite similar, with smooth areas intercalating with rougher ones, and with some cavities being present. In figure 3C, (a detail of a typical particle surface), some pores/cavities can be seen. On the composite particles there are no surface features that might be distinctive for the presence of BG 45S5.

### 3.3. Bioactivity evaluation: chemical analysis

To evaluate the bioactivity of the synthesized particles, both sets of samples – SPLA and SPLA/BG particles – were immersed in a Tris buffer complemented with electrolytes for durations up to 21 days. Figures 4A to 4C display the results of the chemical analysis of Si, Ca and P in immersion solution for the analyzed time points.

![Figure 4](image_url)

**Figure 4.** Si release (A) and Ca (B) and P (C) uptake profiles during 21 days of immersion in Tris buffer with electrolytes’ solution (control). Measurements were made on control solutions (Ctrl) and solutions in which either SPLA or SPLA/BG particles were previously immersed.
As can be seen in figure 4A, BG 45S5 was successfully incorporated into SPLA/BG particles, as seen by the cumulative Si release for the immersion times. As for SPLA particles, no Si release was observed, as would be expected. Together with the Si elution from SPLA/BG particles, variations in Ca and P concentrations were also observed. Regarding calcium, its concentration in solution decreased, indicating that this ion was being deposited at the surface of the particles (figure 4B). Interestingly, this behavior was only observed with the composite particles. Concomitantly, there was also a decrease in phosphate concentration on the solution (figure 4C), as described for calcium. For all the immersion periods, SPLA particles did not produce an uptake of these ions, suggesting absence of bioactive behavior of these microparticles.

3.4. Morphological analysis of immersed SPLA/BG particles

SEM micrographs of the surface of SPLA and SPLA/BG particles immersed for 21 days are shown in figure 5. It is evident the differences between the surface of SPLA (A) and SPLA/BG (B, C) particles, where SPLA (figure 5A) does not evidence Ca-P precipitation oppositely to SPLA/BG particles, which have their surface fully covered by Ca-P nuclei. The morphological analysis of the surface of SPLA/BG particles, combined with the EDS analysis (figure 6) confirms the presence of a layer of calcium phosphate (figure 5B). Higher magnification figure 5C shows that this layer displays the typical morphology of a bone-like calcium phosphate.
Figure 5. SEM images with increasing magnification of the surface of SPLA (A) and SPLA/BG microparticles immersed for 21 days. The surface of SPLA particles does evidence any Ca-P deposition (A), while for SPLA/BG particles the surface is fully covered with a Ca-P layer (B) having the globular structure (C).

EDS analysis (figure 6) was performed to establish the ionic composition of the layer, which is confirmed to be a calcium phosphate by the respective peaks. The presence of chloride is the result of the synthesis in which methylene chloride is employed. There is some residual chloride after washing of the particles.
Figure 6: EDS of SPLA/BG particles immersed for 21 days. Calcium and phosphate peaks confirm the presence of a calcium-phosphate layer at the surface.

The immersed particles were also evaluated by FTIR-ATR (figure 7) and although there is some overlap between the spectra of the polymer and the typical spectra of BG45S5, it is possible to observe the typical P-O band of reacted BG45S5 at 1080 cm⁻¹ at 21 days. Other characteristic BG45S5 bands are not visible due to the overlap with some of the polymer bands.
Figure 7: FTIR spectra for SPLA/BG particles at 0, 14 and 21 days of immersion A) full spectra and B), spectra from 1300-500 cm\(^{-1}\). The arrow indicates the characteristic P-O band that appears when a Ca-P layer is formed on the surface of the material.

Finally, the XRD pattern (figure 8) of the particles was obtained, and upon comparison with the reference HA spectra the appearance of the characteristic peaks of HA on the composite particles is visible after day 14 of immersion.
Figure 8. XRD pattern of SPLA/BG particles immersed for 0, 14 and 21 days in Tris buffer with electrolytes’ solution. The full arrows indicate the main peaks of hydroxylapatite (HA), and dashed arrows indicate secondary peaks as seen at 21 days of immersion. The reference pattern of HA is #3-747.

In figure 8, the XRD patterns of the composite particles are shown after 0, 14 and 21 days of immersion in TE. The arrows indicate the main peaks characteristic of hydroxylapatite. It is seen that at 21 days the main HA peaks as well as some less evident peaks (indicated by the dashed arrows) are present in the composite microparticles. This indicates that at the surface of the composite particles a Ca-P layer similar to hydroxylapatite was formed.

3.5. Short-term cytotoxicity

The results for the short-term cytotoxicity evaluation are present in figures 9 (MTT) and 10 (total protein quantification).
As for total protein quantification, the results are presented as percentages of the control, meaning that the values obtained are in comparison with the 100% proliferation attributed to the control. As can be seen, both materials – SPLA and SPLA/BG - present values close to the one from the control, for both materials above 89%, which is a good indication of their non-cytotoxic behavior.

![Graph](image)

**Figure 9:** Total protein quantification for SPLA and SPLA/BG particles. The values shown are presented as a percentage of the control (cells cultured with culture medium, scored 100%) and for both materials the values are above 88%. No statistically significant differences are observed between both materials (p>0.05).

This result is well correlated with the MTT results, in which both materials present again viability percentages above 86% (figure 10). Some difference is observed between the results for the polymer and composite materials and the T test was performed to assess if significant differences existed between the tested materials for each of the tests, but there are no significant differences between the two materials (p>0.05).
Figure 10: MTT test for SPLA and SPLA/BG particles. The values for both materials are presented as a function of the control (cells cultured with culture medium, scored 100%). Both materials present values above 86%. No statistically significant differences are observed between both materials (p>0.05).

4. CONCLUSIONS

The synthesis of both polymeric and composite starch-bioactive glass particles was successfully achieved, as particles with spherical morphology and a well-defined size range were produced. Immersion in a simulated body fluid for periods up to 21 days showed that BG 45S5 was incorporated into the polymer particles, (as seen by Si release from composite particles) and that its presence rendered the particles bioactive. The bioactive nature of the composite starch-Bioactive Glass 45S5 particles was identified through morphological, chemical and structural analyses. These experiments revealed the presence of a calcium phosphate layer at the surface of SPLA/BG particles.

As for their short-term cytotoxicity, these materials have shown a non-cytotoxic behavior when evaluated in indirect contact tests with a cell line. Both performed tests – MTT and total protein
quantification – have shown to yield identical results, and the tested materials have shown a viability profile close to the one from control cells.

These particles aim to be used as systems for the release of bone growth factors, such as Bone Morphogenetic Proteins (BMPs). By virtue of a combination of bioactive behavior herein assessed and by the ability to encapsulate bioactive agents [16], these microparticles have a great potential for a controlled growth factor release related to bone applications.

Other properties that will also be studied are their bone bonding ability in vivo and their use as injectable bone fillers. Future studies will include the loading of the particles with BMPs to study the incorporation and release of BMP into the particles, in vitro cell culture studies and in vivo studies in small animals.
REFERENCES


Chapter V

Soluble Starch and Composite Starch-Bioactive Glass 45S Particles: Synthesis, Bioactivity and Interaction with Rat Bone Marrow Cells*

Abstract

For many biomedical applications, biodegradable and simultaneously bioactive materials are desired. These materials should at the same time be able to support cell function and co-exist with the organism without triggering a relevant immune response.

In this work the synthesis, as well as the bioactivity evaluation of newly developed polymer soluble potato starch and composite (with Bioactive Glass 45S5) micron size particles is reported. An extremely interesting result is that although with different properties, both polymer and composite particles were able to form a calcium-phosphate layer at their surface, which is a clear indication of their bioactivity.

The cytotoxicity and the ability to support cell attachment and growth of the developed materials was also studied, and both polymer and composite materials were shown to be non-cytotoxic. Preliminary results show that both types of materials were found to allow rat bone marrow cells to attach and to proliferate on their surface and to express osteogenic markers, such as alkaline phosphatase and osteopontin.

* This chapter is based on the following publication
The obtained results indicate that the developed carriers might be used as substrates for cell culture in vitro, in order to form constructs that might be used as a part of a tissue engineering strategy.

1. INTRODUCTION

Materials for bone repair and regeneration are one of the most studied subjects in the biomaterials field. The particular properties of bone render difficult to achieve a satisfactory material that gathers all the desired characteristics including among others mechanical properties, bone bonding ability and biocompatibility. In several applications, biodegradable materials are specially desired, particularly in non-load-bearing applications, where biomaterials used as a bone substitute should be a temporary material serving as a scaffold for bone remodelling\textsuperscript{1-3}. This type of materials must degrade in a controlled fashion into non-toxic products that the body can metabolize or excrete via normal physiological mechanisms\textsuperscript{4,5}.

In vitro models both in terms of biocompatibility and bioactivity evaluation have proven to be valuable tools for testing and screening candidate biomaterials\textsuperscript{1,6-8}. One possible route to in vitro induce bioactivity into polymeric substrates is to use autocatalytic deposition methods and biomimetic routes in order to induce Ca-P layer formation on the surface of the polymers\textsuperscript{9-11}. Another strategy is to combine polymers with bioactive materials such as hydroxylapatite and bioactive glasses\textsuperscript{12,13}.

Since most bone implant materials are implanted into adult bone in direct contact with bone marrow tissue, it is advantageous to use bone marrow cell cultures from adult rats to investigate new implant materials\textsuperscript{14-16}. Bone marrow cells have been reported to proliferate and differentiate, to express bone cell markers and to form mineralized nodules in vitro\textsuperscript{17,18}. In this work we have used such type of cultures aiming at assessing if rat bone marrow cells were capable of displaying this same behavior in the presence of starch and starch-bioactive glass microparticles. In this way,
by isolating and culturing rat bone marrow cells and placing them in contact with potential biomaterials it is possible to gain insights on foreseen behavior of the material in vivo.

2. MATERIALS AND METHODS

2.1. Synthesis of Soluble Starch & Soluble Starch-Bioactive Glass 45S5 particles

Paselli 2 (Pa2), a soluble potato starch chemically modified (AVEBE, Netherlands) was the raw material used. The reinforcement material, Bioactive Glass 45S5 (46.1% SiO$_2$, 24.4% Na$_2$O, 26.9% CaO, 2.6% P$_2$O$_5$, mole%) was obtained from MO-SCI (Rolla, MO, USA) and ground before use to a particle size smaller than 16 µm.

For the synthesis of starch and composite particles, Paselli 2 starch was dissolved in water together with a crosslinking agent, trisodium trimethaphosphate (TSTP). For the synthesis of the composite particles, 30% of the polymer weight of BG 45S5 was mixed with the polymer powder at this step. This solution was then placed under constant vigorous stirring with a top stirrer, to which a mixture of light mineral oil and sorbitan monooleate (SPAN 80) - selected as emulsifying agent - was slowly added. After the formation of the emulsion, sodium hydroxide was added dropwise in order to activate the crosslinking reaction, which proceeded for 6 hours. The emulsion was then destabilized with a mixture of water: acetic acid (1:1) or with 100% ethanol (in case of composite particles to prevent BG 45S5 reactions). Produced particles were collected, extensively washed and then freeze-dried.

2.2. Morphology

The synthesized particles were morphologically characterized by light microscopy and scanning electron microscopy (SEM). The particles were observed and photographed in an optical
microscope with an image acquisition system (high resolution color video camera and Image-Pro Plus analysis software, Media Cybernetics, Silver Spring, Maryland, USA). Scanning Electron Microscopy (SEM) analysis was performed on gold-coated samples in a Leica Cambridge S-360 (Cambridge, UK), and Energy Dispersive Spectroscopy (EDS) was performed on carbon coated samples.

### 2.3. *In vitro* Bioactivity evaluation

Pa2 and Pa2-30%BG particles were tested *in vitro* for their bioactivity in a 0.05 M Tris hydroxymethyl aminomethane-buffered solution complemented with electrolytes typical for plasma (TE). The use of this solution was described previously by Kokubo *et al* 19. Pa2 and Pa2-30%BG particles were immersed in TE at a ratio of 3 mg/ml in a CO₂ incubator at 37ºC. The vials were placed on an orbital shaker at a rotational speed of 150 rpm for up to 21 days without solution exchange. At designed time points (1, 2, 3, 7, 10, 14 and 21 days), the samples were removed from the shaker, the pH of the solution measured and the particles separated from the solution by filtration. The particles were then dried and stored in a desiccator until further analysis. The post immersion solutions were used to perform chemical analysis, namely for changes in Ca, Si and P concentrations, that were evaluated by ICP-OES (Inductively Coupled Plasma- Optical Emission Spectroscopy).

Further characterization of the immersed particles was performed using Fourier Transformed InfraRed Attenuated reflection (FTIR-ATR), Scanning Electron Microscopy/Energy Dispersive Spectroscopy (SEM/EDS) analysis and X-Ray Diffraction (XRD) analysis.
2.4. Short-term cytotoxicity evaluation

For cytotoxicity evaluation by indirect contact with the materials, two different tests were performed: MTT, a viability test widely used to assess the cytotoxicity of a potential biomaterial, and total protein quantification.

The cells used in this study were an immortalized cell line of subcutaneous areolar fibroblasts from mouse origin purchased from Collection of Cell Culture (ECACC, UK, ref. 85011425), designated L929. The cells were grown in monolayer systems in Dulbecco’s Modified Eagle Medium (DMEM) (supplemented with antibiotics and FBS) and used at low passages. The materials were extracted in DMEM culture medium (supplemented as described above) for 24 hours at 60 rpm. After this time period, the culture medium with the materials’ extracts was filtered and added to the cellular monolayer.

2.4.1. MTT

The MTT test is a biochemical test widely used to assess cytotoxicity by measuring cell viability and proliferation in a qualitative way. This biochemical test is based in the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide), (which is water-soluble and has a yellow tonality) by the cell mitochondrial enzyme succinate dehydrogenase, yielding a purple color salt insoluble in water. The salt absorbs at a wavelength of 570 nm and since only living cells have the capability of metabolize the MTT, it gives a measurement of the viable cells. The procedure followed for this test was as described previously. All the materials were tested in 10 replicates for each extract for at least two independent experiments with reproducible results. The results are expressed as a percentage of the control (scored as 100% viability) as mean ± standard errors.
2.4.2. Total Protein Quantification

The method that was used to quantify the total protein uses the Micro BCA Protein Assay Reagent Kit, in which bicinchoninic acid (BCA) is the detection reagent for a Cu complex, which is formed when Cu\(^{2+}\) is reduced by proteins in an alkaline environment\(^{24}\). The purple color product is due to the chelation of two molecules of BCA with one Cu ion. This complex is water-soluble and absorbs at 562 nm, and its optical density is linearly correlated with protein concentration\(^{24}\). The procedure was performed as described in a previous work\(^{23}\).

All the materials were tested in 10 replicates for each extract for at least three separate experiments, with reproducible results. The results are expressed as percentage of the control (scored as 100% viability) as mean ± standard errors.

2.5. Rat Bone Marrow Stromal Cells: isolation and culture

Rat bone marrow cells (RBMC) were isolate and cultured according to the method described by Maniatopoulos et al.\(^{17}\). In brief, femora of 5-week old Wistar rats were removed, cleaned from soft tissue, cut at the epiphyses and then flushed into α-MEM culture medium supplemented with FBS, antibiotics, ascorbic acid, β-glycerophosphate and dexamethasone. The cells were maintained at 37°C, 5% CO\(_2\) for 6 days, with medium change for removing debris.

Meanwhile, a pre-determined amount of Pa2 and Pa2-30%BG particles enough to cover the bottom of a well of a 24-well plate was weighted and placed in non-adherent cell culture plates. The use of this particular type of culture plates is to prevent the quantification of any response coming from cells adhered to the well and not to the materials. Prior to cell seeding, the materials were ethanol-sterilized and pre-incubated for 24 hours in 500 µl of culture medium in the 24-well plates.
The cells were detached using trypsin and seeded at a density of $5 \times 10^4$ cells/well. Two studies were performed – both polymer and composite materials were cultured in two different conditions, culture medium supplemented with and without dexamethasone. Dexamethasone was used – together with ascorbic acid – as a means of inducing the commitment of rat bone marrow cells into the osteoblastic lineage. The absence of dexamethasone in one set of experiments had the aim of assessing if cells cultured on the surface of starch-based particles could commit to the osteoblastic lineage even in the absence of dexamethasone.

The cultures were maintained for 17 days, with periodic evaluations whenever the culture medium was changed (every three days).

### 2.6. Total Protein quantification and Alkaline Phosphatase activity

Total protein quantification was performed as described in section 2.4.2. In each well the culture medium was removed (for ALP quantification) and the particles washed with PBS for total protein quantification. Then 500 $\mu$l of PBS were added followed by 100 $\mu$l of the BCA reagent to each well, and further incubated for 2 hours. The optical densities were measured and total protein (in $\mu$g) was determined using a BSA standard curve.

Alkaline phosphatase (ALP) activity was assayed by the release of p-nitrophenol from p-nitrophenylphosphate as described before $^{25}$.

### 2.7. Immunocytochemistry

The 14-day samples (control particles and particles cultured with cells) were washed twice with 2 ml of PBS 1x (Phosphate Buffered Saline Tablets, Sigma, St. Louis, USA) for 10 minutes. The samples were fixated with 0,5% Glutaraldehyde in PBS 1x (Glutaraldehyde Grade II 25% aqueous
solution, Sigma-Aldrich co., St. Louis, USA) and incubated for 30 minutes at 37°C. After fixation, samples were washed again twice with PBS for 10 minutes.

Afterwards cells were permeabilized through the incubation with a 0.2% solution Triton X-100 in PBS, 2 min at room temperature, followed by a washing step, 3 times with PBS for 5 minutes. The primary antibody used was the mouse monoclonal antibody MPIIIB10 (DSHB, University of Iowa, IA, USA) that was raised against a rat bone extract fraction.

Samples were incubated with the primary antibody in a humidified atmosphere at room temperature with a 1/100 dilution (in a 3% BSA/PBS solution) of the MPIIIB10 anti-Osteopontin antibody for 1 hour. This incubation period was followed by washings (3x) with PBS for 5 min each. For detection of the primary antibody, a 1/100 dilution of the secondary antibody conjugated with a fluorochrome (Alexa Fluor™ 488 goat anti-mouse IgG, Molecular Probes, Leiden, The Netherlands) was added to each lamella, for 1 hour at room temperature and protected from light. After the secondary anti-body incubation the samples were again washed with PBS (3x, 5 minutes each).

The ProLong® Antifade Kit montage medium (ProLong® Antifade Kit (P-7481), Molecular Probes, Leiden, The Netherlands) was prepared and dropped onto the samples and dried for one hour, after which the samples where observed on a fluorescence microscope with a coupled camera/image acquisition system.

3. RESULTS AND DISCUSSION

3.1. Synthesis of polymer and composite starch & starch-Bioactive Glass 45S5

The synthesis of soluble starch Pa2 and Pa2-30%BG 45S5 particles yielded particles with a spherical morphology with varied sizes, up to 500 µm (figures 1A, 1B). The presence of BG 45S5 does not seem to affect both the morphology and the size of the particles (fig. 1B).
Figure 1. Light microscopy images of the synthesised particles, Pa2 (A) and Pa2-30%BG (B).
The appearance of the particles is spherical and no differences in morphology can be attributed to
the presence of BG 45S5 (original magnification 40x).

However, scanning electron microscopy analysis (figure 2) of both Pa2 polymer (A) and Pa-
30%BG composite (B, C) particles shows that at higher magnifications some differences between
polymer and composite particles surface are evident. Regarding the morphology, composite
particles seem to be more regularly spherical than polymer ones. The major differences are
observed at the surface of the particles, where polymeric particles seem to be composed by small
particles that were crosslinked into a bigger network (fig. 2A.).
Figure 2. Representative scanning electron microscope (SEM) images of Pa2 (A) and Pa2-30%BG particles (B) and surface of composite particles (C).

As for composite particles (figs. 2B, 2C) the surface is smoother compared with the one from polymer (fig. 2A) particles. Since the synthesis methodology is the same, these differences can be attributed to the presence of BG45S5 in the composite particles.

3.2. Bioactivity evaluation

The in vitro bioactivity evaluation was based on immersion of the particles in a fluid simulating the blood plasma ionic composition (Tris buffer complemented with electrolytes) up to 21 days. The results for the chemical analysis performed as function of the immersion time are presented in
Figure 3. Figures 3A to 3C display the results of the chemical analysis of Si, Ca and P in immersion solution for the analyzed time points.

**Figure 3.** Si (A), Ca (B) and P (C) profiles during 21 days of immersion in Tris buffer with electrolytes’ solution (control). Measurements were made on control solutions (Ctrl) and solutions in which either Pa2 (Pa) or Pa2-30%BG (PaBG) particles were previously immersed.

As can be seen in figure 3A, BG 45S5 was successfully incorporated into Pa2-30%BG particles, as seen by the cumulative Si release for the immersion times. As for Pa2 particles, no Si release was observed, as expected. Together with the Si, also Ca and P concentration in solution were evaluated for both polymer and composite particles. The variation of calcium in the solution (fig. 3B) is reduced for both Pa2 and Pa2-30%BG particles, when comparing with the control. This indicates that there is only a minor withdrawal of calcium from the solution. As for phosphate (fig. 3C), there are differences between the two materials, since for Pa2 particles the amount of phosphate in solution increases over time. This phenomenon might be explained by the fact that
the synthesis of these materials includes a crosslinking step that uses a phosphate-containing compound, trisodium trimethaphosphate (TSTP), as crosslinking agent. Being these materials biodegradable, we might speculate that the increase in phosphate observed in solution might be due to the scission of less-strong crosslinked starch chains, thus releasing small starch and phosphate groups into the solution. This phosphate release from the structure of the particles occurs via a mechanism independent of the phosphate precipitation that is occurring at the surface of the particles to form a Ca-P layer. The crosslinking mechanism, as represented in figure 4, displays the bonding between the crosslinking agent and starch groups. This phosphate release is most probably higher than the amount of phosphate precipitating at the particles surface, which causes the increase in P amount in solution observed in the figure 3C.

\[
\text{Starch-OH} + \text{Na}_3\text{P}_9\text{O}_{26} \xrightarrow{\text{NaOH}} \text{Starch-O-P-O-Starch} + \text{O}^\prime \text{Na}^+ 
\]

**Figure 4.** Mechanism of starch crosslinking with trisodium trimethaphosphate in sodium hydroxide aqueous (aq) solution\textsuperscript{26}.

As for composite Pa2-30%BG particles, there is a progressive decrease of phosphate in the solution, indicating that this ion was being precipitated at the surface of the particles, giving origin to a bioactive Ca-P layer (figure 3C).

Further analysis by SEM (figures 5, 6) shows that, in fact, both polymer and composite particles display at their surface a deposited Ca-P layer, although more clearly in the composite particles.
Chapter V. Soluble Starch and Composite Starch-Bioactive Glass 45S Particles: Synthesis, Bioactivity and Interaction with Rat Bone Marrow Cells

Figure 5. Pa2 particles after 21 days of immersion (A,B). In image B, a higher magnification showing the morphology of the Ca-P layer covering the surface of the particles.

Figure 6. Pa2-30%BG particles covered with a Ca-P layer after 14 days of immersion (A) and surface close-up after 7 days of immersion (B).

For Pa2 particles, there is a layer of Ca-P deposited at the surface, confirmed by EDS (figure 7A), and P seems to be present in higher amounts at the particles’ surface than Ca. This result can be explained by the presence of P in the starch network, which does not allow determining the Ca-P ratio of the layer. Previous works have shown that materials with terminal P groups are able to form an apatite layer when soaked in SBF without induction time and at a higher growth rate than for other groups \(^{27}\). In the present work, the presence of phosphate in the starch particle
network might induce the Ca deposition and consequent Ca-P formation at the surface of the particles, since it is known that the apatite induction is facilitated by increasing concentrations of either Ca or P\textsuperscript{28}.

As for the composite particles (fig. 7B.), EDS analysis also reveals the presence of Ca-P layer formation at the surface of the particles, which is already visible at 7 days of immersion (fig. 6B, SEM image), confirming the stronger bioactive character of the composite particles.

**Figure 7.** Energy Dispersive X-ray analysis for Pa2 polymeric particles (A) and for Pa2-30\%BG 45S5 composite particles (B).

FTIR analysis of Pa2-30\%BG 45S5 particles (figure 8) reveals differences in the spectra of non-immersed particles (0 days of immersion) and after 21 days of immersion. It is visible the enhancement of the intensity of bands at 1100-1050 cm\textsuperscript{-1}, which correspond to the P-O stretch, as well as the enhancement of the C=O bond at 1740 cm\textsuperscript{-1}.
Figure 8. FTIR spectra of Pa2-30%BG 45S5 particles for 0, 14 and 21 days of immersion. • Indicates the C=O bond and ■ indicates the P-O stretch.

The nature of this Ca-P layer was also investigated regarding its crystalline nature by X-Ray Diffraction, as presented in figure 9.
Figure 9. X-Ray Dispersion of Pa2-30%BG particles at 0, 7, 14 and 21 days of immersion. Main peaks are observed at 26 and 32 at 2θ angle values, although several secondary peaks are present at other values. The main peaks are typical for HA 90432 JCPDS file.

By comparison with the 90432 Hydroxylapatite JCPDS standard, it is possible to observe that Pa-30%BG particles after 7 days of immersion already present one of the HA main peaks. It is known that the crystallization of insoluble salts involves the formation of metastable precursor phases \(^{27}\), so with increasing immersion time, the layer of Ca-P is growing and rearranging, and at 14 days other typical HA peaks are visible that, at 21 days, correspond with a great number of less intensity peaks present in the HA 90432 standard.
Figure 10. X-Ray Dispersion of Pa2 particles at 0, 10, 14 and 21 days of immersion. HA peaks are observed at 32 and 46 degrees. * indicates the material peak, and • indicates the major peaks appearing at 10, 14 and 21 days of immersion.

For Pa2 particles the XRD pattern reveals that the layer of Ca-P is growing with time, by the disappearance of the material peak (*) and the appearance of crystalline peaks at 27, 32, 46 and 57 degrees (•).
3.3. Short-term cytotoxicity

The results for the short-term cytotoxicity evaluation are present in figures 11 (total protein) and 12 (MTT). The results are presented as percentages of the control both for MTT and total protein, meaning that the values obtained are in comparison with the 100% proliferation attributed to the control.

As it can be seen, both polymer and composite materials present total protein values close to the one from the control. The results obtained for both materials are above 89%, which is a good indication of their clearly non-cytotoxic behavior.

![Graph showing total protein quantification for Pa and Pa2-30%BG 45S5 particles. The values shown are presented as a percentage of the control (cells cultured with culture medium, scored 100%) and for both materials the values are above 89%. No statistically significant differences are observed between both materials (p>0.05).]

Figure 11. Total protein quantification for Pa and Pa2-30%BG 45S5 particles. The values shown are presented as a percentage of the control (cells cultured with culture medium, scored 100%) and for both materials the values are above 89%. No statistically significant differences are observed between both materials (p>0.05).

This result is well correlated with the MTT results, in which both materials present viability percentages above 87% (figure 12). Some difference is observed between the results for the polymer and composite materials and the t test was performed to assess if these differences were
significant. The result of the t test shows that no significant differences are found between the two materials (p>0,05).

![Graph showing MTT test for Pa2 and Pa2-30%BG 45S5 particles. The values for both materials are presented as a function of the control (cells cultured with culture medium, scored 100%). Both materials present values above 87%. No statistically significant differences are observed between both materials (p>0,05).]

**Figure 12.** MTT test for Pa2 and Pa2-30%BG 45S5 particles. The values for both materials are presented as a function of the control (cells cultured with culture medium, scored 100%). Both materials present values above 87%. No statistically significant differences are observed between both materials (p>0,05).

### 3.4. Total Protein and ALP quantification

In figure 13 is displayed the evolution of the protein content during 17 days of culture of the particles with rat bone marrow cells.
Figure 13. Total protein content for 17 days of culture. Pa W – polymeric particles cultured with rat bone marrow cells with dexamethasone in culture medium; Pa W/O - polymeric particles cultured with rat bone marrow cells without dexamethasone in culture medium; PaBG W – composite particles cultured with rat bone marrow cells with dexamethasone in culture medium; PaBG W/O - composite particles cultured with rat bone marrow cells without dexamethasone in culture medium.

It is visible that cells are able to adhere to the particles surface, and that protein content reaches a steady state after 7 days of culture, which may indicate that cells are differentiated, since proliferation and differentiation are described as uncoupled processes. Regarding alkaline phosphatase activity, figure 14 shows its tendency after normalization for the total protein content. For composite particles the ALP highest activity is observed at day 7 of culture with particles, as for polymeric particles this peak is reached after 14 days of culture. This may indicate that the presence of BG 45S5 might influence the fate of these cells earlier than polymeric materials.
Figure 14. Alkaline phosphatase activity per protein content. Pa W – polymeric particles cultured with rat bone marrow cells with dexamethasone in culture medium; Pa W/O - polymeric particles cultured with rat bone marrow cells without dexamethasone in culture medium; PaBG W – composite particles cultured with rat bone marrow cells with dexamethasone in culture medium; PaBG W/O - composite particles cultured with rat bone marrow cells without dexamethasone in culture medium.

3.5. Immunocytochemistry

As for the assessment of osteopontin expression of rat bone marrow cells cultured in the presence of Pa2 and Pa2-30%BG particles, with or without dexamethasone supplemented to the culture medium, the results are observed in figures 15 and 16.
Figure 15. Immunocytochemistry for osteopontin in Pa2 particles after 14 days of culture. A – Pa2 particles autofluorescence; B – Control, Pa2 particles (without cells) treated with anti-osteopontin antibody; C – Pa2 particles cultured with rat bone marrow cells treated with osteopontin antibody. For both conditions – presence and absence of dexamethasone – A, B and C were identical, for this reason only one image per condition is shown.

The results for Pa2 particles after 14 days in culture with rat bone marrow cells, clearly show that osteopontin is being expressed, which indicates both that cells have adhered to the materials and that the cells were being able to express bone markers. Another interesting result is that we found no differences in osteopontin detection between cultures with and without dexamethasone. It could be speculated that this result might be due to the influence of the materials on the rat bone marrow cells fate, which would lead to a commitment of the cells to the osteogenic lineage regardless of the presence of dexamethasone. However, to confirm this hypothesis further studies regarding other osteogenic markers, namely osteonectin, osteocalcin, collagen I and Runx-2/cbfa-1 expression must be performed.

As for Pa2-30%BG particles, although both their autofluorescence and control show some staining, which is due to the presence of BG 45S5, there is a considerable difference in the fluorescence of the particles cultured with rat bone marrow cells, allowing to state that osteopontin is being expressed. As already seen for Pa II particles, again no differences were found between
culture conditions (absence or presence of dexamethasone). For this type of particles, the presence of BG45S5 might play a role in cell differentiation, since it is known from the literature that this type of bioactive glass is capable of enhancing cell differentiation. However, further studies must be performed to assess the validity of this hypothesis.

![Image](image1.png)

**Figure 16.** Immunocytochemistry for osteopontin in Pa2-30%BG particles after 14 days of culture. A – control, Pa2-30%BG particles autofluorescence; B – control, Pa2-30%BG particles (without cells) with anti-osteopontin antibody; C – Pa2-30%BG particles cultured with rat bone marrow cells treated with osteopontin antibody. For both conditions – presence and absence of dexamethasone – A, B and C were identical, for this reason only one image per condition is shown.

### 4. CONCLUSIONS

It is believed that there is a correlation between the *in vitro* bioactivity of a material and its ability to bond to bone. In this way, immersion of the materials in a fluid simulating body ionic composition and the eventual formation of a calcium-phosphate layer at the surface of the materials denotes a possible *in vivo* bioactive behavior. In this work, both polymer (Pa2) particles and composite (Pa2-30%BG) particles were shown to form *in vitro* an apatite-like Ca-P layer at their surface, that was analyzed and shown to have a different nature in both materials.
Short-term cytotoxicity testing shows that cells cultured with both polymer and composite leachables remain clearly viable. Preliminary studies on the ability of these particles to support attachment and growth of undifferentiated rat bone marrow cells have shown that cells do adhere to the materials, and that they express osteopontin both in the presence or absence of dexamethasone in the culture medium.

These results constitute the basis of further studies involving these materials and primary cultures of bone marrow cells, both in standard cell culture conditions and in a HARV (High Aspect Ratio Vessel) bioreactor, where other osteoblastic markers will be evaluated both by biochemical tests and gene expression profiles, in order to establish differences between both conditions. The aim is to move to use these particles as carriers for cells, forming tissue engineering constructs in appropriate bioreactor culture conditions.
REFERENCES


CHAPTER VI
Chapter VI

The effect of starch and starch–bioactive glass composite microparticles on the adhesion and expression of the osteoblastic phenotype of a bone cell line

ABSTRACT

There is a clear need for the development of microparticles that can be used simultaneously as carriers of stem/progenitor cells and as release systems for bioactive agents, such as growth factors or differentiation agents. In addition, when thinking on bone tissue engineering applications, it would be very useful if these microparticles are biodegradable and could be made to be bioactive. Microparticles with all those characteristics could be cultured together with adherent cells in appropriate bioreactors to form in vitro constructs that can then be used in tissue engineering therapies.

In this work we have characterized the response of MC3T3-E1 pre-osteoblast cells to starch-based microparticles. We evaluated the adhesion, proliferation, expression of osteoblastic markers and mineralization of cells cultured at their surface. The results clearly show that MC3T3-E1 pre-osteoblast cells adhere to the surface of both polymeric and composite starch-based microparticles and express the typical osteoblastic marker genes.

*This chapter is based on the following publication:
GA Silva, OP Coutinho, P Ducheyne, IM Shapiro, RL Reis, The effect of starch and starch–bioactive glass composite microparticles on the adhesion and expression of the osteoblastic phenotype of a bone cell line, Biomaterials, 2006, accepted for publication
Furthermore, the cells were found to mineralize the extracellular matrix (ECM) during the culture period.

The obtained results indicate that starch-based microparticles, known already to be biodegradable, bioactive and able to be used as carriers for controlled release applications, can simultaneously be used as carriers for cells. Consequently, they can be used as templates for forming hybrid constructs aiming to be applied in bone tissue engineering applications.

1. INTRODUCTION

To try to regenerate bone has been a major goal of tissue engineering research. A promising approach combines the use of scaffold materials together with autologous site-specific cells. In this way, it may be possible to construct a hybrid material that can repair an osseous defect. For this strategy to be successful, materials need to be generated that exhibit adequate physical and chemical properties, and at the same time enhance cell adhesion, proliferation and differentiation. An ideal substrate for the synthesis of bone should be able to promote the expression of the osteoblastic phenotype as well as provide a template for bone deposition\(^1\). Furthermore, it is desirable for excellent scaffold materials to release bioactive molecules in a controlled fashion such that cell adhesion, proliferation and other cellular functions are enhanced.

Starch-based materials were shown to possess a wide range of properties that support their potential for biomedical applications. Coupled with their biodegradable nature\(^2,3\), the ability to be processed by diverse methods\(^4-6\) and into diverse shapes (3-D porous scaffolds, microparticles, bone cements)\(^7-11\), render these materials very attractive to be used as scaffolds. Blends of starch with different synthetic polymers have been studied for several biomedical applications, such as bone scaffolds\(^12-17\) and drug release applications\(^7,18\). Three-dimensional porous scaffolds based on starch-based materials have been shown to be biocompatible and to possess excellent \textit{in vivo} behavior\(^12,14,16,19\).
In this work, we have focused on the production of starch-based microparticles, which are bioactive\textsuperscript{20} and can release, in a sustained manner, molecules of biological interest\textsuperscript{19}. However, to be used for biomedical purposes, their behavior regarding critical cellular functions such as adhesion, proliferation and maintenance of a defined phenotype needs to be well known. If osteoblast-like cells can adhere and grow at the surface of the starch-based microparticles these substrates could be used for: (i) non-load bearing applications or as part of a 3-D construct; (ii) cultivating anchorage-dependent cells in a dynamic bioreactor and (iii) encapsulating bioactive molecules in the microparticles and simultaneously growing cells at the surface of the microparticles that would release encapsulated growth factors to stimulate proliferation and differentiation of adherent cells. The concept underlying (iii) is illustrated in figure 1. Microparticles incorporating bioactive agents (such as growth factors) combined with progenitor cells would serve simultaneously as carriers for cells and as release systems. The bioactive agent incorporated into the microparticles would be released and act locally on the cells of the construct. Cell proliferation and differentiation would occur, giving origin to a hybrid cell-material construct. After moving the construct to the \textit{in vivo} location, the biodegradable nature of the microparticles would allow them to be replaced by newly formed tissue.

The aim of the present study was to evaluate the ability of starch-based biodegradable microparticles to support cell adhesion, viability and phenotypic expression of osteoblastic markers by MC3T3-E1 cells. In order to assess this, we have used cells of pre-osteogenic lineage and cultured them for periods up to 14 days at the surface of both polymer and composite starch-based microparticles.
Figure 1. Schematic drawing that illustrates the concept of using starch-based microparticles as support materials, carriers for cells and release systems. Microparticles incorporating bioactive agents, such as growth factors, are combined with progenitor cells and cultured in vitro. The growth factor being released from the matrix of the microparticles acts on the cells in the construct, stimulating them to proliferate and further differentiate. This in vitro construct could then be used in vivo, and over time the particles would fully degrade and be replaced by new tissue.

2. MATERIALS & METHODS

2.1. Materials

Starch-based polymer (SPLA, a blend comprised of 50% wt corn starch and 50% wt polylactic acid) and composite (SPLA/BG, comprised of SPLA and 30% Bioactive Glass 45S5 granules, with a composition of 46.1% SiO$_2$, 24.4% Na$_2$O, 26.9% CaO, 2.6% P$_2$O$_5$, mole%) microparticles were produced as described in a previous work$^{20}$. Particle sizes between 210-350 µm were selected over smaller ones, due to the following reasons: compared with smaller sizes, they have larger surface area, which might allow the adhesion of higher number of cells per particle and yield bigger hybrid constructs.

The cell line used in this work – MC3T3-E1, subclone 4, derived from fetal mouse calvaria, was purchased from ATCC (American Tissue Cell Collection). The cells were cultured in DMEM
medium supplemented with 10% FBS and 1% of antibiotics (penicillin - streptomycin) and cultivated in standard tissue culture conditions (37°C, 5% CO₂).

2.2. Evaluation of SPLA and SPLA/BG cytotoxicity

The possible cytotoxicity of the produced particles was screened as described previously. Briefly, SPLA and SPLA/BG microparticles were placed in 15 ml conical tubes with DMEM culture medium (at a ratio of 1g/5ml) and subjected to constant agitation (60 rpm) at 37°C for 24 h, in order for the materials to release eventual leachables (formation of extracts from the materials). As an extraction control, DMEM culture medium was placed under the same conditions. After 24 h the extracts were filtered through a 0.45 µm pore size filter. A monolayer of cells at 80-90% confluence was then treated with the extraction medium for 72 h. After this time period the viability of the cells was evaluated by the MTT assay. All the materials were tested in 10 replicates for each, in at least three independent experiments. The results were expressed as a percentage of the control (scored as 100% viability) and derive from at least 3 independent experiments.

2.3. Cell adhesion to the surface of starch-based microparticles

The ability of MC3T3-E1 cells to adhere to the surface of starch-based microparticles was evaluated for up to 6 h. To determine the adhesion efficiency, 1 µl volume of microparticles was added to a suspension of 2x10⁵ cells. The cells were allowed to adhere for 30 min, 1, 2 and 6 h. After each time period, the microparticles were washed to make them free of non-adherent cells and transferred to a new vial. A volume of trypsin, enough to cover the microparticles, was added to detach the adherent cells, which was then neutralized by the addition of complete culture medium. Cells were then counted in a Neubauer chamber. Adhesion efficiency was determined as
the percentage of adhered cells versus the total number of cells seeded. Data reports results from 3 independent experiments.

2.4. Cell seeding on the surface of starch-based microparticles

Before being used in any cell culture experiments, starch-based microparticles were sterilized with 70% ethanol, allowed to dry and then hydrated in DMEM culture medium prior to cell seeding. A volume of 10 µl of microparticles was then added to a suspension of 5x10^5 MC3T3-E1 cells. The microparticles and cells were mixed and centrifuged for 30 s at 100 rpm. After a maximum period of 12 h, the microparticles with seeded cells were transferred to 6-well plates containing 40 µm pore size cell strainers (BD Falcon, Bedford, MA, USA). The cell strainers prevented particle loss during culture medium changes.

The cells were cultured for 14 days and evaluated for cell proliferation, enzyme activity, gene expression and an end-point assay for mineralization by Alizarin Red staining.

2.5. MC3T3-E1 viability assessment using confocal laser microscopy

Viability of MC3T3-E1 cells adhered to starch-based microparticles was assessed by confocal laser microscopy (Inverted Confocal Microscope, Olympus FloView, Melville, NY, USA). For this purpose was used a viability fluorescent dye – CellTracker Green CMFDA (Molecular Probes, Eugene, OR, USA). This dye diffuses through the cell membranes and once inside the cell, the CellTracker, containing a chloromethyl group that reacts with thiols, is transformed into a cell-impermeable fluorescent dye-thioether adduct. Only living cells have the ability to allow this reaction to occur, and this principle was used to determine the distribution and viability of cells adhered to the surface of starch-based microparticles.
The medium from the samples (SPLA and SPLA/BG microparticles with adhered cells) was aspirated and replaced by a 1:1000 dilution of CellTracker in serum-free DMEM. After 30 min the working solution was removed and replaced with complete culture medium. After a second 30 min incubation period, the samples were analyzed in a laser confocal microscope, with an excitation laser of 517 nm. Images for SPLA and SPLA/BG samples were obtained by stacking of 20 µm planar slices.

2.6. MC3T3-E1 DNA quantification

DNA content, as a means of evaluating proliferation, was measured using the PicoGreen dsDNA kit (Molecular Probes, USA). PicoGreen dsDNA reagent is an ultra sensitive fluorescent nucleic acid stain for the quantification of double stranded DNA (dsDNA) in solution. At each time point in culture, cell strainers were removed and the contents (particles with cells) washed with isotonic saline solution and centrifuged. A minimal volume of 0.1 N NaOH was added to release DNA from the cells. An aliquot of the DNA suspension was added to 10x Tris-EDTA buffer, to which was then added the PicoGreen reagent (previously prepared in 10x Tris-EDTA buffer) in a 1:1 vol:vol, and fluorescence measured in a microplate reader at 485 and 535 nm excitation and emission wavelengths, respectively. Lambda DNA was used as standard. The data presents results of at least three independent experiments.

2.7. Evaluation of alkaline phosphatase activity

Alkaline phosphatase was measured using the AttoPhos AP fluorescent Substrate System (Promega, Madison, WI, USA). AttoPhos Substrate (2´-[2-benzothiazoyl]-6´-hydroxybezothiazole phosphate [BBTP]) is cleaved by alkaline phosphatase to produce inorganic phosphate (P_i) and
the alcohol, 2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole (BBT). This enzyme-catalyzed conversion of the phosphate form of AttoPhos Substrate to BBT is accompanied by an enhancement in fluorescence. Samples (microparticles and adhered cells) were transferred to a 1.5 ml tube, centrifuged at 14000 rpm for 1 min, the supernatant (culture medium) was discarded and the pellet was suspended in 1ml of ice-cold 0.9% NaCl solution in 3 mM Tris-HCl (pH 7.4) and again centrifuged at 14000 rpm for 1 min. The pellet was solubilized in 500 µl of a 0.9% NaCl and 0.2% Triton X-100 solution. 100 µl of cell suspension were added to 200 µl of Attophos reagent, mixed for 15 min, after which 100 µl were loaded into each well of a 96-well plate and fluorescence was read at 430 nm excitation and 595 nm emission.

2.8. RNA extraction

Total RNA was extracted using the TRIzol reagent (Life Technologies, Gaithersburg, MD). In order to isolate the RNA, chloroform was added to the samples (microparticles with adherent cells), followed by precipitation with isopropyl alcohol.

Samples were transferred to a 1.5 ml tube, centrifuged at 14000 rpm for 2 min and the supernatant was removed. Then, 1ml of TRIzol was added to the pellet, and samples were re-suspended about 10 times to lyse the cells. Afterwards, 250 µl of chloroform were added to the samples and the mixture was vortexed. The mixture was then centrifuged at 14000 rpm for 10 min, at 4°C. The top aqueous fraction was collected into a RNase free tube, to which 2 volumes of isopropanol were added. The solutions were mixed and again centrifuged at 14000 rpm, for 30 min at 4°C. The supernatant was discarded and 1 ml of ethanol was added to wash the pellet by centrifugation at 14000 rpm, 6 min, at 4°C. The supernatant was discarded and the samples were air dried for approximately 10 min. The RNA samples were then resuspended in 40 µl of mili-Q sterile water. The concentration and purity of the RNA were measured in a UV spectrophotometer.
at 260 nm and by calculating the A260/A280 ratio, respectively. The integrity of the RNA was assayed by electrophoresis of the samples in a 1% agarose gel.

2.9. RT-PCR analysis of osteoblastic markers

RT-PCR was performed using a one step procedure. The method was optimized to an amount of 200 ng of RNA. The PCR reaction components were added in the following order: water (ddH₂O), Reaction Mix (Invitrogen, Carlsbad, CA, USA), primers, sample RNA and the enzyme, Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The samples were then run in a program with the following parameters shown in table I. GADPH, a housekeeping gene, was run as the control. After completion of the PCR, the products were electrophoresed in a 1.5% agarose gel, together with a 100 bp DNA Ladder (Promega, Madison, WI, USA) and visualized in a Kodak UV imager.

Table I. RT-PCR program details.

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2.10. Mineralization assay: Alizarin Red

The Alizarin Red mineralization assay was performed using the method described by Bodine et al.²², with modifications. Samples were washed with PBS, and subjected to a fixative 10% v/v
solution of formaldehyde in PBS for 15 min. After removal of fixative, samples were washed twice with excess water and covered with Alizarin Red (AR) solution, followed by gentle agitation in an orbital shaker for 20 min. The AR solution was then removed and the samples washed four times with mili-Q water. The samples were observed in an optical microscope with a coupled SPOT camera.

2.11. Alizarin red staining extraction

The protocol for extraction of the Alizarin Red staining was adapted from Gregory et al.\textsuperscript{23}. 500 µl of acetic acid (10% v/v) were added to the stained samples, incubated for 30 min at room temperature with mild shaking. The monolayer was then scrapped off the plate, transferred to a 1.5 mL tube and vortexed for 30 s. The slurry was overlaid with 300 µl of mineral oil to prevent evaporation and heated at 80°C for 10 min. Then the samples were centrifuged for 15 min at 14000 rpm. 300 µl of the supernatant was transferred to a 1.5 mL tube and mixed with 100 µL of a 10% (v/v) solution of ammonium hydroxide. 100 µL aliquots were transferred to a 96-well plate and the absorbance read at 430 nm. At least 3 independent experiments were performed, each one with triplicates.

2.12. Statistical analysis

Results are expressed as mean ± standard deviation. Differences between experimental results were analyzed according to a Student t-test, with p<0.05 considered statistically significant.
3. RESULTS

3.1. Cytotoxicity screening of starch-based polymer and composite microparticles

Testing for eventual cytotoxicity is the first step in the examination of any material proposed for biomedical use. The ISO MEM extraction test evaluates extracts/leachables of the materials in a cell culture system. Cell viability is determined by a biochemical assay (MTT) compared with standard tissue culture conditions as maximum viability. In a previous work, we have described the evaluation of the cytotoxicity of SPLA and SPLA/BG microparticles using a fibroblast cell line where the materials were shown to be non-cytotoxic for that specific cell line.

Figure 2 displays the results of this test for MC3T3-E1 cells and starch-based microparticles. It is clear that leachables from both materials – polymer and composite - allow cells to remain viable, exhibiting values close to the control. No statistically significant differences were found between both materials (p>0.05). These results confirm that the tested materials are not cytotoxic and can be further evaluated for their potential for biomedical applications.

![Figure 2](image)

**Figure 2.** MC3T3-E1 cells treated with extracts from polymer (SPLA) and composite (SPLA/BG) microparticles display viability values similar to the control (cells fed with regular DMEM) as evaluated by the MTT assay. The results for each material is shown as percentage of the control. There are no statistically significant differences between materials, as analyzed by the t test, for a confidence level of 95% and n=3.
3.2. Adhesion of MC3T3-E1 cells to starch-based microparticles

The first hours of contact between cells and materials are critical, since it is well-known that for anchorage dependent cells, adhesion to a substrate has to occur within few hours, otherwise the cells will lose their viability\textsuperscript{24}. In addition to anchoring cells, adhesive interactions activate various intracellular signaling pathways that direct cell viability, proliferation, and differentiation\textsuperscript{25-27}.

Thus, to establish the ability of a biomaterial to serve as a substrate for cell culture, the adhesion efficiency of cells needs to be evaluated prior to the establishment of the long-term culture system.

In this work, we evaluated the adhesion of MC3T3-E1 cells to starch-based microparticles and figure 3 presents the results for SPLA and SPLA/BG compared to cell adhesion to tissue culture polystyrene (TCPS), so far considered the ideal material for cell adhesion.

![Graph](image)

**Figure 3.** MC3T3-E1 cell adhesion to tissue culture polystyrene (TCPS), SPLA polymeric and SPLA/BG composite microparticles after 6 hours. Cell adhesion was evaluated by removal of adhered cells from the surface of the microparticles and count in a hemocytometer. The $t$ test result revealed statistically significant differences between all conditions (TCPS vs SPLA, $n=3$, $p=0.0016$; TCPS vs SPLA/BG, $n=3$, $p=0.0147$; SPLA vs SPLA/BG, $n=3$, $p=0.0158$).

As expected, cell adhesion to TCPS reached the highest value from all conditions (90% of all seeded cells). Cell adhesion to SPLA polymer microparticles was about 40% of the total number of
seeded cells; for composite microparticles, this value reached 60%. These values can be considered very good for biodegradable materials with no prior surface modification. Statistical analysis revealed significant differences between SPLA and TCPS \( (p=0.0016) \), SPLA/BG and TCPS \( (p=0.0147) \) and between SPLA and SPLA/BG \( (p=0.0158) \). The difference in adherence values probably reflects the variation in material properties, in terms of chemistry, surface charges, reactive groups and roughness.

We also evaluated the thiols status (which provides a mean of evaluating cell viability) of cells adhered to the surface of both polymer and composite microparticles using confocal laser microscopy after 1 and 2 days in culture.

**Figure 4.** Laser confocal microscopy of MC3T3-E1 cells stained with CellTracker green (A) and (D), TCPS; (B) and (E), SPLA; (C) and (F), SPLA/BG microparticles. (A), (B) and (C) show cells adhered to TCPS, SPLA and SPLA/BG microparticles after 1 day in culture, respectively. (D), (E) and (F) cells adhered to TCPS, SPLA and SPLA/BG microparticles after 2 days in culture.
respectively. Cell viability and distribution in all conditions is evidenced by the green fluorescence. Images (B), (C), (E) and (F) were obtained by stacking of 20 µm planar slices to build a three dimensional image. Original magnification for A and D: 80x; for all others 40x.

Figures 4(A) and (D) show the fluorescence of cells adhered to TCPS after one and two days, respectively. It is noticeable that they are spread over the surface, and this behavior is also observed at day 2 (D). The fluorescence of the cells is high, indicating that the cells are in a reduced thiol status and hence viable. Cells are clearly attached to both SPLA (B and E) and SPLA/BG (C and F) microparticles at day 1, although some of the cells present a round morphology and appear to be clumped together. However, at day 2 of culture, cells are well adhered to the microparticles (E, F), and completely cover the surface of some of the microparticles of the aggregate (F). Again this is a rather good result, not typical at all for biodegradable materials.

Another interesting result is the formation of 3-D aggregates between microparticles and cells (Figures 5A and 5B). These aggregates are maintained throughout the culture period. Although these systems do not present sufficient mechanical properties to be used per se in load bearing applications, this behavior is desirable in dynamic cell culture conditions, such as those using bioreactors. Preliminary results have shown that these aggregates can be placed and cultured in the NASA-approved HARV bioreactor for up to 3 weeks without disaggregating the constructs. This seems to point to the eventual formation of tissues in vitro that can be functional when implanted in vivo, as a means of promoting the regeneration of the damaged tissue.
Chapter VI. The effect of starch and starch-bioactive glass composite microparticles on the adhesion and expression of the osteoblastic phenotype of a bone cell line

Figure 5. Images of aggregates formed between cells and microparticles, similar to the ones in figure 3, at 14 days of culture. The images show 3-D aggregates formed during the in vitro culture, and these aggregates were maintained throughout the whole culture period. (A) SPLA microparticles and (B) SPLA/BG microparticles. Original magnification 10x.

3.3. MC3T3-E1 proliferation at the surface of starch-based microparticles

Cell proliferation at the surface of polymer and composite microparticles was measured by determining the increase in DNA content up to 14 days (Figure 6).

Figure 6. MC3T3-E1 cell proliferation in TCPS, SPLA and SPLA/BG microparticles. Extrapolation from DNA to cell number was performed using a standard curve of DNA from known
cell numbers. The proliferation of the cells is reduced for both polymer and composite microparticles when compared to TCPS. Nevertheless, cells are able to remain viable and proliferate at the surface of the carriers. Statistical analysis was performed through the $t$ test. * and ** indicate significant differences between TCPS and SPLA, SPLA/BG, respectively. Statistically significant differences between SPLA and SPLA/BG (●) found only for values at 14 days.

MC3T3-E1 cells were able to proliferate at the surface of both polymer and composite microparticles at similar rates until 7 days of culture. Cells on TCPS proliferated at a ratio of $6 \times 10^5$ cells/day. In contrast, the rates on SPLA and SPLA/BG were $1.2 \times 10^5$ cells/day and $9.1 \times 10^4$ cells/day, respectively. These statistically significant differences might be explained based on the surface of the materials. Starch-based microparticles, being composed of a polymeric mixture of PLA and starch, have at their surface domains that are richer in PLA and others richer in starch. Therefore this creates some surface heterogeneity that might influence cell adhesion.

Differences between both starch-based particles are only observed after 7 days of culture, where cells at the surface of polymeric microparticles increase their number by approximately 10% more than cells cultured at the surface of composite microparticles, and this difference was found to be statistically significant ($p=0.0068$).

3.4. Alkaline phosphatase activity

The data obtained from the measurement of Alkaline Phosphatase activity showed very low enzyme activity and no obvious differences among the materials and TCPS. MC3T3-E1 cells failed to express alkaline phosphatase, however, other works in literature$^{28-32}$ have shown these cells to be a good model of the osteoblastic lineage. Failing to express significant levels of alkaline phosphatase activity, nevertheless, does not impart the role of these cells as a model for the
osteogenic pathway. Further results in this study show that these cells are in fact committed and able to maintain the osteoblastic lineage.

3.5. Expression of osteoblastic marker genes

Markers of the osteoblastic phenotype – osteopontin, osteocalcin, collagen type Iα and the transcription factor Runx-2, were analyzed by RT-PCR (figure 7).

![Figure 7. RT-PCR analysis of osteoblastic genes expressed by MC3T3-E1 cells. Cells were cultured on TCPS, SPLA and SPLA/BG microparticles for 14 days, then evaluated by RT-PCR. Transcripts evaluated were osteopontin, osteocalcin, collagen type Iα and Runx-2. GADPH, a housekeeping gene, was run as a loading control.]

On TCPS all the markers were expressed, although levels of osteopontin (OP) and Runx-2 were low. On SPLA, the level of expression of Runx-2 and collagen type Iα was similar to TCPS. In contrast to TCPS and SPLA, on SPLA/BG composite microparticles, there was a higher level of
expression of all of the transcripts, which indicates that composite microparticles clearly enhance the expression of osteoblastic markers.

### 3.6. Alizarin Red Staining

To examine the mineralization potential of MC3T3-E1 cells on TCPS, SPLA and SPLA/BG, we stained the cultures with Alizarin Red. Figure 8 shows that both SPLA and SPLA/BG microparticles enhance mineralized nodule formation compared to cells cultured on a TCPS surface. Cells cultured on the standard tissue culture surface (TCPS) only showed Alizarin Red staining comparable to the one found in SPLA microparticles after 4 weeks of culture (data not shown).

**Figure 8.** Alizarin Red staining for TCPS (A), SPLA (B) and SPLA/BG (C) microparticles, evidencing the higher mineralization levels for both polymer and composite microparticles compared with TCPS. Original magnification 100x.

When quantitatively analyzing, on a cell basis, the amount of deposited calcium (figure 9), cells cultured on standard conditions (TCPS) showed statistically significant lower levels when compared to polymeric (SPLA, \( p=0.002 \)) and composite (SPLA/BG, \( p<0.0001 \)). On SPLA/BG
microparticles calcium was also found to have significantly higher values than those observed for SPLA samples (p=0.0068).

Figure 9. Alizarin Red dye quantification for TCPS (control), SPLA and SPLA/BG microparticles. After staining the dye was extracted and quantified in a spectrophotometer. The OD values were normalized for the cell number. Statistically significant differences were found between all conditions (TCPS vs SPLA, p=0.002; TCPS vs SPLA/BG, p<0.0001; SPLA vs SPLA/BG, p=0.0068, for n=3.

4. DISCUSSION

Starch-based biodegradable microparticles were evaluated in this study for their ability to allow cell adhesion, proliferation and expression of the osteoblastic phenotype of cells cultured on their surface. One of the drawbacks of using biodegradable materials for many biomedical applications is the fact that their biodegradable nature challenges the adhesion of cells to their surface\textsuperscript{33}. While for many materials low cell adhesion efficiency creates the need for surface modification\textsuperscript{34}, in this study, cell adhesion to the surface of non-surface modified starch-based microparticles reached values up to 60%. Considering that the microparticles were not subjected to any kind of surface
modification to enhance cell adhesion, cell adhesion values are likely due to the presence of high number of hydroxyl (OH) groups at the surface of the microparticles due to the starch component of the material. For other starch-based materials, the adhesion of cells has been shown to be higher for materials with lower oxygen content\textsuperscript{35}, although hydroxyl groups/high ratio of oxygen to carbon have been shown to enhance cell adhesion\textsuperscript{36,37}. Studies of the surface chemistry by X-ray Photoelectron Spectroscopy (XPS) could help elucidate this result. In addition, the presence of Bioactive Glass 45S5 might also contribute to the differences in cell adhesion observed between polymer and composite microparticles. Bioactive Glass 45S5 has been extensively studied and it has shown, both \textit{in vitro} and \textit{in vivo}, adequate properties for osseous applications\textsuperscript{38-45}. We have studied previously the production of composite SPLA/BG 45S5 microparticles\textsuperscript{20}, and observed that the surface morphology was not altered by the presence of the ceramic component. However, Bioactive Glass 45S5 might alter the surface chemistry of the microparticles and this can be the basis for the differential cell adhesion of MC3T3-E1 pre-osteoblastic cells to the surface of polymer and composite microparticles.

It was previously suggested\textsuperscript{46} that surfaces that show good cell attachment at early time points do not necessarily promote cell proliferation or differentiation. In the present case, cell proliferation on the surface of both polymer and composite microparticles is significantly lower compared with proliferation values on TCPS. These rates can be due to (i) a heterogeneous surface, where domains richer in the starch component alternate with domains richer in the PLA component; (ii) degradation of the material, that causes removal of potential adhesion points for cells and (iii) formation of 3-D aggregates between microparticles and cells that may hinder the proliferation of cells in the inner areas of the aggregate. In this scenario, proliferation would be localized to the outer surface of the aggregate. A possible solution to overcome this drawback makes use of dynamic cell culture conditions, in which the circulation of nutrients and waste products removal is enhanced\textsuperscript{47-49}.

With these results, we would expect the osteoblastic phenotype of the cells to be affected. However, RT-PCR results show that cells are able to maintain their phenotype when cultured at
the surface of the microparticles. Although only a residual amount of alkaline phosphatase was detected, gene expression constitutes a proof of the osteoblastic phenotype. Lineage allocation to osteoblasts has been shown to be controlled at the transcriptional level by Runx2, an osteoblast-specific transactivation factor. In the present study Runx2 expression was observed for both polymer and composite microparticles, its expression being enhanced in composite SPLA/BG microparticles. The higher level of expression observed for cells cultured at the surface of composite microparticles was also observed for the other osteoblastic markers.

The differences in gene expression observed between polymer and composite materials are most likely due to the presence of Bioactive Glass 45S5. Other works have shown that the ionic products of bioactive glass dissolution can stimulate genes concerned with osteoblastic metabolism and bone homeostasis. We have already shown before that composite SPLA/BG microparticles are bioactive, and this bioactivity was shown in vitro by ionic dissolution followed by precipitation of calcium phosphate at the surface of the microparticles. In the present case, MC3T3-E1 cells seem to be stimulated by composite microparticles to express higher level of gene transcripts that denote their osteoblastic nature, and we assume the presence of Bioactive Glass and the ionic dissolution phenomenon to be responsible for this result.

Well correlated with the results for gene expression are the results of the Alizarin Red staining, evidencing mineralization. For polymer and composite microparticles, mineralization was greatly enhanced when compared with the one for TCPS, both quantitatively and temporally. Cells in TCPS did attain similar levels of mineralization to those found for cells cultured on the surface of polymer microparticles, but with a two-week delay.

In summary, although cell adhesion had lower values and proliferation rates are slower for polymer and composite microparticles when compared to control (TCPS), cell-specific functions were enhanced. Altogether, there was a more robust expression of the osteoblastic genes and an increase in critical mineralization activity. When SPLA and SPLA/BG are compared, the composite microparticles present a superior array of desirable features. These include bioactivity (as
previously shown), higher values for cell adhesion as well as enhanced expression of osteoblastic markers and calcium deposition.

5. CONCLUSIONS

In a previous study we have proven, through the formation of a calcium-phosphate layer at their surface, the in vitro bioactivity of starch-based microparticles. This characteristic allows one to infer about the osteoconductive and osteoinductive behavior of these materials. Additionally, these materials were shown to be able to incorporate and release bioactive molecules, such as dexamethasone and growth factors. We now show another role of these microparticles, namely the enhancement of the expression of the osteoblastic phenotype by pre-osteoblastic cells cultured at their surface.

The data herein presented confirms that starch-based microparticles (both polymer and composite) are capable of supporting the in vitro growth and maturation of osteoblast-like cells. Differences between polymer and composite microparticles include higher cell adhesion values, mineral deposition and gene expression for the latter.

In addition to the previously described roles these systems can play, this study further confirms that these starch-based microparticles could be used in bone tissue engineering strategies incorporating bioactivity, controlled release and cell support properties.
REFERENCES

Chapter VII

Entrapment Ability and Release Profile of Corticosteroids from Starch-Based Microparticles

Abstract

We previously described the synthesis of starch-based microparticles that were shown to be bioactive (when combined with Bioactive Glass 45S5) and non-cytotoxic. To further assess their potential for biomedical applications such as controlled release, three corticosteroids with a similar basic structure – dexamethasone (DEX), 16α-methylprednisolone (MP) and 16α-methylprednisolone acetate (MPA) were used as models for the entrapment and release of bioactive agents.

Dexamethasone, 16α-methylprednisolone and 16α-methylprednisolone acetate were entrapped into starch-based microparticles at 10% wt/wt of the starch-based polymer and the loading efficiencies, as well as the release profiles, were evaluated. Differences were found for the loading efficiencies of the three corticosteroids, with DEX and MPA being the most successfully loaded (82 and 84%, respectively), followed by MP (51%). These differences might be explained based on the differential distribution of the molecules within the matrix of the microparticles.

*This chapter is based on the following publication:
Furthermore, a differential burst release was observed in the first 24 hours for all corticosteroids with DEX and MP being more pronounced (around 25%), while only 12% of MPA was released during the same time period. While the water uptake profile can account for this first stage burst release, the subsequent slower release stage was mainly due to degradation of the microparticle network. Differences in the release profiles can be explained based on the structure of the molecule, since MPA, a more bulky and hydrophobic molecule, is released at a slower rate when compared with DEX and MP.

In this work it is shown that these carriers were able to sustain a controlled release of the entrapped corticosteroids over 30 days, which confirms the potential of these systems to be used as carriers for the delivery of bioactive agents.

1. INTRODUCTION

Due to the considerable advantage of their clearance from the body after the release of therapeutic agents, biodegradable polymers are among the most widely used materials for controlled drug delivery applications. Starch-based polymers have been studied mainly by Reis et al. for a wide range of bone related applications, ranging from tissue engineering scaffolds, to bone cements and drug delivery systems. These materials display a set of features that support their potential in the biomedical field, such as natural origin, good mechanical properties, good biological performance and the possibility of tailoring their properties according to the foreseen application. Recently, we have described the use of a blend of starch and polylactic acid (SPLA) to synthesize microparticles (polymer and composite with Bioactive Glass 45S5) with a defined size range that were found to be bioactive and non-cytotoxic. A potential application for these developed microparticles is as carriers for bioactive agents in controlled release applications.
There are two basic general strategies to develop polymeric matrices for delivery systems: the use of hydrophobic matrices that can release encapsulated drugs as a result of their bioerosion or biodegradation, or the use of hydrogel matrices, that can swell and retain large volumes of water, thus allowing diffusion of the drug. The combination of these two strategies (by using a blend of hydrophobic-hydrophilic polymers, such as polylactic acid and starch) might allow for the synthesis of a carrier that combines the best features of each material. Factors influencing release rate include drug molecular size and loading efficiency into the polymer, polymer composition and molecular weight and the dimensions, shape and crystallinity of the matrix.

Considering all these aspects, the entrapment of bioactive molecules within starch based microparticles and subsequent release profile were evaluated in order to assess the potential of these micron size systems to be used as carriers for drug delivery. For this purpose, three different corticosteroids were chosen as model bioactive agents. Corticosteroids have a widespread use in clinical practice, due to their broad range of anti-inflammatory activities, being used in affections such as acute respiratory distress, degenerative diseases, as well as immunosuppressors in organ transplantation. Dexamethasone has a high estimated potency, being used for several applications, in which ocular ones have a major importance. Dexamethasone delivery systems for eye-delivery, as well as for preventing stent restenosis, have been documented. It has also been widely used in in vitro cell culture where differentiation of bone marrow cells into the osteoblastic lineage is to be achieved. Methylprednisolone is used similar to dexamethasone, finding applications in endocrine and rheumatic disorders as adjunctive therapy for short-term administration, collagen diseases during exacerbation or as maintenance therapy, as also in several dermatological diseases (psoriasis, dermatitis, Stevens-Johnson syndrome).

In the present work we describe the ability of starch-based microparticles to act as carriers for drug delivery applications, by means of entrapping and evaluating the release profiles of three corticosteroids, namely dexamethasone, 16α-methylprednisolone and 16α-methylprednisolone acetate.
2. MATERIALS & METHODS

2.1. Materials

A blend of starch and polylactic acid herein referred as SPLA (50% cornstarch-50% PLA, by weight) was used as the raw material. The PLA used in this blend was PLA 4040, 94% L-Lactide from Cargill-Dow (Minneapolis, MN, USA), and the blended material was supplied by Novamont (Novara, Italy). More information on this material can be found elsewhere. Methylene chloride (Sigma, St Louis, MO, USA) and polyvinyl alcohol 87-89% hydrolyzed (Aldrich, Milwaukee, WI, USA) were used for the production of the microparticles. Dexamethasone ≥ 98% (HPLC grade, Sigma, St Louis, MO, USA), 16α-Methylprednisolone ≥ 99% and 16α-Methylprednisolone acetate ≥ 99.3% (both in house produced) were used as the bioactive molecules for entrapment and release studies. All other chemicals used were reagent grade (Panreac, Barcelona, Spain) except methanol that was HPLC grade (Riedel-de-Haën, Sigma/Aldrich, Seetze, Germany).

2.2. Synthesis of starch-based microparticles loaded with corticosteroids

The synthesis methodology for SPLA microparticles was as described previously. Briefly, starch-polylactic acid blend (SPLA) was mixed with the steroid (10% weight of polymer) and dissolved in methylene chloride. This solution was then emulsified with a stirring 0.5% polyvinyl alcohol (PVA) solution for up to 4 hours in a top stirrer at a speed of 660 rpm/min. The microparticles were then washed and separated from the reaction solution by filtration. The reaction solution was stored for quantification of the non-loaded corticosteroid. Steroid-loaded microparticles were freeze-dried and stored in a desiccator until further use. This procedure was performed separately for each of the corticosteroids used, namely dexamethasone (DEX), 16α-
Methylprednisolone (MP) and 16α-Methylprednisolone acetate (MPA). Values of 2.5%, 5% and 10% wt/wt (dexamethasone/polymer) were tested in order to determine the ratio of bioactive agent to polymer that achieved the highest encapsulation efficiency.

At least three independent experiments were performed for each of the corticosteroids with reproducible results.

2.3. Physicochemical characterization of SPLA microparticles

Starch-based microparticles – unloaded, steroid-loaded and after a 30-day release period - were gold coated and analyzed by scanning electron microscopy (SEM) using a Leica Cambridge S-360 model (Cambridge, UK) to assess morphological features and differences between conditions.

In order to assess changes in the microparticles between pre and post release, Fourier Transformed InfraRed Attenuated reflection (FTIR-ATR) was performed. Spectra were recorded at least at 32 scans with a resolution of 2 cm\(^{-1}\) in a FTIR spectrophotometer (Perkin-Elmer 1600 Series). All the samples were analyzed using a single reflection ATR system (MKII Golden Gate\textsuperscript{TM}, Specac) with a diamond crystal (angle of incidence = 45°; active sampling area 0.8×0.8mm; depth of penetration 20 µm).

2.4. Degradation and water uptake of SPLA microparticles

Measuring the weight loss of the microparticles assessed the degradation behavior, and the water uptake behavior measured by the capability of the microparticles to incorporate water in their matrix (swelling), being both parameters studied over a period of 90 days. For this, SPLA microparticles were weighted and immersed in a saline phosphate buffer solution (PBS) at
physiological pH (pH 7.4) at a ratio of 1mg/1ml, and placed into a water bath at 37ºC with constant agitation (60 rpm).

After pre-determined time periods (up to 30 days for water uptake, 90 days for weight loss) the vials containing the immersed microparticles were centrifuged at low speed for particle deposition and the supernatant was removed for further measurements of degradation products. The remaining wet microparticles were weighted for water uptake measurements. Water uptake was determined using the following equation

\[
\% \text{ Water uptake} = \left( \frac{m_w - m_i}{m_i} \right) \times 100,
\]

where \(m_i\) is the initial weight and \(m_w\) is the wet weight of the sample. After each time period the samples were dried at 37ºC and the final mass of the samples weighted to determine the weight loss according to the following equation

\[
\% \text{ Weight loss} = \left( \frac{m_f - m_i}{m_i} \right) \times 100,
\]

where \(m_f\) is the final weight of the dry sample and \(m_i\) is the initial weight of the sample. Triplicates were performed for each sample at each time period and the results are shown as mean ± standard deviation.

2.5. Determination of DEX, MP and MPA loading efficiency and release profile

2.5.1. Loading efficiency

The loading efficiency for the different steroids was determined by measuring the amount of corticosteroid remaining in the reaction medium (unloaded) where the microparticles were
synthesized. Aliquots of 1 ml of the reaction medium were taken and the amount of DEX, MP and MPA present in each sample measured by High Performance Liquid Chromatography (HPLC) as described in section 2.6. The results were obtained from three independent experiments run in duplicate with reproducible results and the mean value is reported.

The loading efficiency was calculated from the following formula:

\[
\text{Loading efficiency (\%)} = \frac{\text{CS}_i - \text{CS}_r}{\text{CS}_i} \times 100\%
\]

where CS\textsubscript{i} is the initial amount of corticosteroid to be loaded and CS\textsubscript{r} the amount of corticosteroid remaining in the reaction medium (unloaded), where microparticles were synthesized.

2.5.2. In vitro release studies

\textit{In vitro} release studies were conducted in phosphate buffer saline solution (PBS), 0.1M, pH 7.4. Pre-weighted corticosteroid-loaded microparticles were immersed in sterile PBS at a ratio of 5 mg/ml under mild agitating conditions, at 37\textdegree C. At pre-determined time points, PBS aliquots were sampled for further quantification of the released corticosteroid. The results were obtained from two independent experiments run in duplicate with reproducible results and the mean value is reported.

2.6. HPLC analysis

Samples were extracted 3 times with a mixture of hexane and ethyl acetate (1:1), to selectively remove the steroids. The extracts were combined and evaporated under a nitrogen flow. The residues were re-dissolved in methanol and subjected to analysis by HPLC.
The HPLC system consisted of an auto-injector Midas Injector Spark, and a Beckman System Gold Solvent Module 126 coupled to a Detector Module 166. The separation of the samples was performed in a PurospherStar RP-18e column (250x4.5 mm, 5µm particle size; Merck, Germany), using methanol and water as solvents. The following gradient was utilized: 50% methanol for 10 min, 30% methanol-70% water for 25 min. The flow rate was 1 ml/min and the detection wavelength used was 254 nm. Quantification of DEX, MP and MPA was performed using the internal standard method using Triamcinolone (Lederle, New York, USA) as the reference compound.

3. RESULTS & DISCUSSION

3.1. Synthesis of starch-based microparticles with encapsulated DEX, MP and MPA

Figures 1 and 2 show representative scanning electron microscope images of the unloaded and corticosteroid-loaded microparticles, respectively. The synthesis method yielded corticosteroid-loaded microparticles with morphologies similar to unloaded microparticles (figure 1). Therefore, the entrapment of the steroids does not seem to produce significant changes in the morphology of the microparticles.
Figure 1. Representative image of unloaded SPLA microparticles showing the two different morphologies observed in each batch of microparticles, one displaying a rough surface and the other a smoother surface.

For both unloaded and loaded microparticles, two different surfaces can be found, namely microparticles with a rougher surface (2A) and microparticles with a smoother surface (2C). For the rougher surface microparticles, a porous surface is observable at higher magnifications (fig. 2.B.), which greatly enhances the surface area of the microparticle, when compared to the surface of a smoother microparticle (fig. 2.D.), where no pores are visible.

Staining with iodine solution was performed in order to distinguish between starch and PLA phases, since iodine is known to bind to starch, yielding a dark blue color. Iodine staining revealed that the rough surface is due to the starch phase (stained as dark blue areas), and the smooth surface due to the PLA phase (results not shown). This morphological feature, combined with the hydrophobic PLA and hydrophilic starch natures, can greatly influence the properties of the microparticles in terms of degradation and release profiles.
Figure 2. Morphology of the corticosteroid-loaded microparticles. In A, the particle presents a rough surface, with apparent porosity (B, detail of surface). In C and D, images of a microparticle displaying a smoother surface. Both morphologies are representative of the microparticles with different loaded corticosteroids.

3.2. Water uptake and degradation profiles of the microparticles

The release of a drug from a matrix is primarily controlled by diffusion of the drug through the polymer due to its swelling in aqueous medium, and the dissolution rate of the drug, being erosion of the polymer an additional but however still very important factor. For biodegradable polymers, it is essential to recognize that degradation is a chemical process, whereas erosion is a physical phenomenon dependent on dissolution and diffusion process. In this way, knowledge of the water uptake capability and degradation of polymeric materials is of great importance in studies
regarding their use for controlled release applications. For SPLA microparticles, the hydration and weight loss profiles are shown in figures 3 and 4, respectively.

The establishment of the water uptake profile might give insights on the release mechanism, since in the case of polymers drug diffusion is primarily controlled by swelling of the polymer matrix. In the present case, SPLA microparticles have the ability to swell up to values as high as 400% of its weight, after 12 hours of soaking, reaching the equilibrium after one week in PBS solution, with a value around 300% (figure 3). After the initial release stage controlled by swelling of the matrix, subsequent release is primarily controlled by degradation of the polymer (chemical process)\textsuperscript{31}. As so, monitoring the weight loss up to 90 days assessed the degradation of SPLA microparticles.

![Graph showing water uptake profile](image)

**Figure 3.** Water uptake profile of SPLA microparticles as determined by immersion in a phosphate buffer saline solution for up to 30 days (n=9).
The weight loss profile of the microparticles in figure 4 can be described by a reduced initial weight loss until 48 hours, followed by a period spanning from 48 to 500 hours where about 20% of weight is lost. Then a reduced decrease in weight is observed up to 90 days.

**Figure 4.** Weight loss profile of SPLA microparticles as determined by immersion in a phosphate buffer saline solution up to 2160 hours (90 days) (n=9).

The first step in the degradation of the microparticles occurs mainly through scission of small size starch chains into oligosaccharides, as assessed by the appearance of reducing sugars in solution (data not shown), up to 500 hours. The rate of degradation then decreases, since the cleavage of larger starch chains is to occur, being this coupled to the eventually slower degradation of the PLA phase. Further details on the degradation mechanisms of starch-based biomaterials can be found elsewhere \(^{40-42}\).
3.3. Entrapment efficiency

The entrapment/loading efficiency was determined by measuring the amount of corticosteroid remaining on the reaction media after the synthesis of the corticosteroid-loaded microparticles. Using HPLC to analyze aliquots of the reaction media (where corticosteroid-loaded microparticles were synthesized) it was possible to quantify for unloaded corticosteroid. This value was then subtracted to the initial amount of corticosteroid to be loaded, allowing obtaining the efficiency of drug loading (through the formula described in section 2.5.1. of Materials and Methods).

In order to optimize the loading, it was studied if varying the percentage of drug to be encapsulated would lead to a variation in the loading efficiency, in particular for DEX. Values of 2.5%, 5% and 10% were entrapped in the microparticles and the loading efficiency evaluated.

As shown in figure 5, the higher the initial amount of drug to be entrapped, the higher the encapsulation efficiency. The highest encapsulation efficiency was attained when 10% wt drug/wt polymer was used, with a value of 82%. This drug to polymer ratio was chosen for subsequent studies with all the corticosteroids.

![Graph showing influence of DEX initial amount on loading efficiency](image)

**Figure 5.** Influence of DEX initial amount on its loading efficiency. The amount (in g) of loaded DEX is shown as labels.
Table I displays the results of the loading efficiencies for each corticosteroid entrapped in the starch-based microparticles.

**Table I.** Loading efficiencies for the three corticosteroids into the starch-based microparticles at a loading of 10% wt/wt drug/polymer. Measuring the amount of corticosteroid remaining on the reaction media where the microparticles were formed and subtracting it to the initial amount of corticosteroid allowed determining the efficiency of loading.

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Loading efficiency</th>
</tr>
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<tbody>
<tr>
<td>Dexamethasone (DEX)</td>
<td>82%</td>
</tr>
<tr>
<td>16α-Methylprednisolone (MP)</td>
<td>51%</td>
</tr>
<tr>
<td>16α-Methylprednisolone acetate (MPA)</td>
<td>84%</td>
</tr>
</tbody>
</table>

From table I it is clear that DEX and MPA have the highest encapsulation efficiencies, with 82 and 84%, respectively. The less successful encapsulation was attained for MP, with 51% loaded into the microparticles.

The differences in the loading efficiencies might be explained by the structure of the corticosteroids used (figure 6). The basic structure is the same, being the only difference between these molecules their side groups, which in DEX is a fluoride atom covalently bonded, while for MPA is the acetate group. As for MP, it can be considered the basic structure when compared with the two other molecules.
Figure 6. Chemical structure for DEX (a), MP (b) and MPA (c).

MPA is a bulkier molecule than DEX or MP. This bulkiness could facilitate MPA entrapment within the polymer network that would result in the highest entrapment efficiency (Table I). This result correlates well with the data obtained from FTIR, where the presence of peaks due to drug loading is evident for DEX (fig. 7) and also for MPA (fig. 8), which can explain the high loading achieved. The entrapment efficiency is lower for MP (51%, Table I), which by FTIR is shown by the absence of MP characteristic peaks in the spectrum (fig. 9).
Figure 7. FTIR spectra of DEX, SPLA, SPLA-DEX loaded and SPLA-DEX released microparticles. The black dots (•) indicate bands present in the microparticles due to loading of dexamethasone. After 30 days of release, the same bands are still visible, although a reduction in the intensity is observed.

It is visible from the analysis of the FTIR spectra that for DEX (figure 7) and MPA (figure 8) there were some changes in the spectra due to drug loading in the SPLA microparticles, where bands characteristic of these corticosteroids appeared in the spectra of the loaded microparticles. This might be due to the fact that some drug becomes trapped on/close to the surface of the polymer matrix during the manufacturing process, which is in accordance with the described in the literature for other systems \(^{43,44}\). As for MP (figure 9), such changes were almost absent in the FTIR spectrum of the loaded microparticles, which as said before, can explain the lower loading efficiency when compared with DEX and MPA.
For the MPA-loaded microparticles there was a reduction in the intensity of the peaks due to the corticosteroid loading after 30 days of release, which seems to support the hypothesis that the corticosteroid trapped at the surface of the microparticles has been released at the initial stages of release. Regarding DEX, there was some reduction in the intensity of the bands due to corticosteroid loading, but nevertheless they remain visible after 30 days of release.

**Figure 8.** FTIR spectra of MPA, SPLA, SPLA-MPA loaded and SPLA-MPA released microparticles. The asterisks (*) indicate bands present in the microparticles due to loading with MPA. After release, these bands are almost absent from the structure of MPA-loaded microparticles.
Microparticles loaded with MP show only minor changes in the structure when compared with unloaded microparticles. The same observation is evident in the spectrum of microparticles after 30 days of MP release.

3.4. *In vitro* release profile for DEX, MP and MPA

The *in vitro* release profiles for dexamethasone (A), 16α-methylprednisolone (B) and 16α-methylprednisolone acetate (C) are shown in figure 10.
Chapter VII. Entrapment Ability and Release Profile of Corticosteroids from Starch-Based Microparticles

**Figure A:**
- Graph showing the % Cumulative Release of DEX over hours.

**Figure B:**
- Graph showing the % Cumulative Release of MP over hours.
Figure 10. Release profiles as determined by HPLC quantification. A - DEX, B – MP and C – MPA. The results are shown as percentage of cumulative release. Right-insert graphs show the profile for the first 12 (MP, figure B) and 24 (DEX, MPA, A and C, respectively) hours of release. A burst release occurs for all steroids, although for MPA this effect is less pronounced (12%) when compared with DEX (27%) and MP (28%).

Analysis of the release profiles indicates that all the corticosteroids are released with an initial burst phase, followed by a slower release, typical of a first order release kinetics. This is in accordance with what is described for other biodegradable polymers. The release profile is similar for DEX and MP, with 27% and 28% of release for the first 24 hours, respectively. This initial burst is then followed by a slower release until 750 hours (30 days), where 45% of entrapped DEX and 40% of MP are released. For MPA, it is observed a reduced burst effect (around 12% for the first 24 hours) when compared with the other two molecules. This initial release is then followed by a slower release that reaches 30% of the entrapped MPA by 30 days.
Interestingly, an incomplete release pattern is observed for all corticosteroids, which was also observed for chitin/PLGA 50:50 microspheres. This confirms the potential of this system for prolonged release of entrapped drugs.

The initial burst phase can be explained by the fact that the swelling of the material controls the initial release stages (fig. 3, water uptake, first 12 hours), and the drug is thus released by diffusion through the channels created by water penetration. At this stage, degradation/weight loss does not play a significant role in release, since by 24 hours only 2% of the weight is lost (fig. 4, weight loss). The second, slower release period can be attributed to the weight loss, since this phenomenon is only evident after 48 hours, in which a decrease in weight of around 20% is observed until 500 hours.

The degradation and corticosteroid release of the microparticles after 30 days (750 hours, figure 11) produces changes in the morphology of the microparticles, evidenced by pores in the surface of the microparticles. Nevertheless, the microparticle matrix structure is still maintained, which could account for the drug still remaining to be released.

Figure 11. Representative images of the morphology of corticosteroid-loaded SPLA microparticles after 30 days of release in a phosphate buffer saline solution. Some pores are evident in the surface of the microparticles, but the matrix structure is still maintained.
The water uptake and weight loss do not account however, for the differences observed among the corticosteroids. Comparing the release profiles of MP (fig. 10.B.) and MPA (fig. 10.C.) the differences in the release profile can be attributed to the structure of the molecule. Although MPA loading efficiency was higher than the one for MP (Table I), since the former has a more bulky structure than MP, its release was expected to be slower than the one for MP. Moreover, the presence of the acetate group in MPA (fig. 6.C.) renders to this molecule a more hydrophobic character than MP (considered the basic structure) (fig. 6.B.) and thus MPA affinity towards the aqueous moiety is significantly reduced. This same explanation is applicable to the difference between DEX and MPA release: although they possess a similar loading efficiency (82 and 84%, respectively), DEX was released faster and at a higher percentage than MPA, consistent with a molecule size effect.

When DEX is compared to MP, difference in the molecule bulkiness (fig. 6.A. and 6.B.) cannot explain the differences in the loading efficiencies. Furthermore, it does not seem to have any effect in the release profiles. Importantly, when all the release profiles are compared, it should be emphasized that the release into the medium is inversely correlated with increasing bulkiness and hydrophobicity of the molecules.

4. CONCLUSIONS

In a previous work by using a blend composed of starch and polylactic acid (SPLA), we demonstrated the ability to form non-cytotoxic microparticles within a defined size range. In this work we evaluated the potential of these microparticles for release applications, by studying the loading and release of corticosteroids as model bioactive agents. We successfully encapsulated dexamethasone and two other corticosteroids – methylprednisolone and methylprednisolone acetate - with the encapsulation efficiencies varying between 51% and 84%. These differences
might be explained by the structure of the molecules and the differential distribution of the molecule within the microparticle structure.

The in vitro release profiles showed that these starch-based systems are capable of sustained release of the entrapped steroids up to 30 days. The burst release observed in the first 24 hours was lower for MPA when compared with DEX and MP. After this period, release proceeds for over 30 days, with DEX and MP being released up to 45% and MPA up to 30%. While the water uptake can account for the initial burst release, degradation can account for the subsequent release stage. The remaining loaded corticosteroids could be released at later stages, and this release is likely controlled by the degradation of the microparticles.

From these studies we suggest that starch-based microparticles could be used as carriers for bioactive agents, and this investigation constitutes a basis for future entrapment and release studies using other bioactive agents, such as growth factors (BMPs, VEGF, PDGF) or others relevant for biomedical applications.
REFERENCES


Chapter VIII

Starch-Based Microparticles as Vehicles for the Delivery of Active Platelet-Derived Growth Factor*

Abstract

In a previous work we described the use of starch-based microparticles as vehicles for the controlled release of corticosteroids. The goal of the present work is to evaluate the potential of these microparticles to incorporate and release Platelet-Derived Growth Factor (PDGF). The loading efficiency and release profile were evaluated, and PDGF was incorporated into and released from the matrix of starch-based microparticles. The release profile shows that there was rapid release of PDGF in the first 24 h, after which there was a slow but constant release for up to 8 weeks. The maintenance of the PDGF biological activity after incorporation and release was evaluated by its mitogenic effect over osteoblastic cells, and it was shown to be comparable to that of exogenous PDGF. This proves that the incorporation and release did not affect the biological activity of the growth factor.

The results clearly demonstrate that starch-based microparticles are suitable vehicles for the incorporation and release of growth factors. Combined with previous results, these materials can enhance the regenerating potential of tissue engineering hybrid constructs.

* This chapter is based on the following publication:
1. INTRODUCTION

In Tissue Engineering, there has been considerable interest in using growth factors (GF) to enhance regeneration and achieve faster repair.

Among growth factors of potential interest is Platelet-Derived Growth Factor (PDGF). PDGF is a polypeptide dimer with a molecular mass of 30,000. It is a well-known mitogen for cells of mesenchymal origin\textsuperscript{1-4}, which was initially isolated from platelets and subsequently found to be synthesized by a variety of skeletal and non-skeletal cells\textsuperscript{5}. This growth factor has three different isoforms, each a dimeric combination of two distinct, but structurally related peptide chains: AA, AB and BB\textsuperscript{6-8}. These dimeric isoforms are differently expressed in various cell types. They also differ in the specific binding to two different cell receptors: PDGFr-\textbf{α} and PDGFr-\textbf{β}\textsuperscript{6-8}.

PDGF isoforms have been found to be mitogenic for several cell lineages an effect apparently mediated by the MAP (Mitogen Activated Protein) kinase pathway\textsuperscript{9}. Bone regeneration and repair, like other tissue healing processes, involve a complex cascade of events, including chemotactic, proliferative and maturational phases\textsuperscript{2}. PDGF is a growth factor known to act in earlier phases, promoting cell chemotaxis\textsuperscript{10-12} and proliferation\textsuperscript{5}, but not maturation/differentiation of osteoblast cells\textsuperscript{13,14}. Initially, chemotaxis of osteoblast precursors to the site of bone regeneration is mediated by growth factors such as PDGF. In addition, PDGF has been shown to stimulate migration and to increase the proliferation rate of osteoblasts \textit{in vitro}\textsuperscript{15,16}, reduce alkaline phosphatase activity\textsuperscript{16} and inhibit bone matrix formation\textsuperscript{17}.

In tissue engineering strategies, the combination of scaffold materials with cells specific for the defect site is an attractive means of creating a tissue engineering construct capable of promoting regeneration. The supplementation with growth factors is intended to improve the efficacy of bone formation\textsuperscript{18}. In this case, by incorporating the growth factor within the matrix of the scaffold, the latter serves not only as a support material but also as a release device. Biodegradable scaffolds are most desirable for such applications, as they serve only temporarily, being replaced as new tissue is formed.
However, during processing of the scaffold the bioactive molecules can often be inactivated due to solvents or processing temperature. To avoid such limitations, we propose the bioactive molecule (in this case, a growth factor) to be incorporated into starch-based, biodegradable, micron size particles, produced at room temperature and by a simple emulsion method. As part of a new strategy, illustrated in figure 1, the produced particles would then be combined with a cell population (stem/progenitor cells) to form a 3-D structure. The release of the GF from the microparticles would have as direct targets the cells at the surface of the materials, acting to enhance functions such as proliferation and differentiation. The biodegradable nature of the microparticles would allow them to be replaced by newly formed tissue in the \textit{in vivo} location at a later stage. This strategy could be particularly useful when using cells isolated from the patient, that usually exist in limited supply.

The objective of the present study is to determine the capability of starch-based microparticles to incorporate PDGF and release the incorporated GF in a sustained manner. Since starch-based microparticles have already been shown to be bioactive through formation of a Ca-P layer \textit{in vitro} \cite{19}, to release other biologically active molecules in a sustained way \cite{20} and to support cell adhesion, proliferation and phenotypic expression of osteoblastic markers \cite{21}, we explore herein their use as carriers for growth factors.
**Figure 1.** Cartoon illustrating the strategy proposed for generating hybrid (bioactive molecule-scaffold-cell) constructs. The polymer and growth factor (GF) are combined to form a scaffold microparticle entrapping the growth factor within its matrix (1.). These microparticles are then combined with appropriate cells (progenitor or lineage specific) (2.) and cultured for an appropriate time period (3.). During culture, the growth factor incorporated in the microparticles is released by diffusion and by degradation of the scaffold matrix (4.), thus stimulating the adhered cells to
proliferate, differentiate and form mineralized matrix (5.). Ultimately, the scaffold totally degrades and is completely replaced by new tissue.

2. MATERIALS AND METHODS

2.1. Materials

A blend comprised of 50% wt cornstarch and 50% wt polylactic acid, by weight [PLA 4040, (94% L-Lactide) was obtained from Cargill-Dow, Minneapolis, MN, USA, and the blended material was supplied by Novamont (Novara, Italy)] designated SPLA was used as the raw material. Human recombinant Platelet-Derived Growth Factor-BB (hPDGF-BB) was purchased from R&D Systems, Minneapolis, USA.

The cell line used in this work – MC3T3-E1, subclone 4, derived from foetal mouse calvaria, was purchased from ATCC (American Tissue Cell Collection). The cells were cultured in DMEM medium supplemented with 10% FBS and 1% of antibiotics (penicillin - streptomycin) and cultivated in standard tissue culture conditions (37°C, 5% CO₂).

2.2. Synthesis of PDGF-SPLA microparticles

Briefly, and as described earlier¹⁹, SPLA powder was partially dissolved in 10 ml of methylene chloride for approximately 15 min. The SPLA methylene chloride solution was then added dropwise to 200 ml of 0.5% poly vinyl alcohol solution and continuously stirred for 3.5 h at room temperature in order to evaporate/extract the organic solvent and harden the microparticles. The resultant microparticles were sieved by appropriate size meshes, washed multiple times with
distilled water and freeze-dried.

Microparticles incorporating Platelet-Derived Growth Factor (PDGF) were produced by co-encapsulating PDGF with bovine serum albumin (BSA) (fraction V, Fisher, USA). BSA is commonly used as a carrier\(^2\) for the encapsulation and protection of bioactive molecules from the harsh organic solvents, which can cause inactivation of the bioactive molecules.

BSA and PDGF were mixed prior to addition to the SPLA in methylene chloride solution at a ratio of 1:20 000 of PDGF:BSA (i.e. 10 µg PDGF in 200 mg BSA). The resultant microparticles were sieved into 3 different size ranges: <40 µm, 40-210 µm and >210 µm. Particles with size >210 µm were chosen for this study, following studies in which this same size range was used for the culture of osteoblastic cells\(^21\). Microparticles incorporating BSA alone were also produced, as a control. Throughout this study the following controls were used: (i) SPLA microparticles (GF-free) and (ii) BSA-loaded microparticles.

### 2.3. Loading efficiency

The loading efficiency was defined as the ratio of PDGF incorporated in the SPLA microparticles to the initial amount of PDGF. To this end, 20 mg of PDGF-SPLA microparticles were dissolved overnight in 1 N NaOH and PDGF loading efficiency was measured by ELISA (as described in section 2.5). BSA loading efficiency from BSA-loaded SPLA microparticles was measured by the Bradford method for protein quantification (Bio-Rad, Hercules, CA, USA), by measuring the absorbance at 284 nm on a spectrophotometer against known BSA concentrations.
2.4. Morphological characterization

Scanning Electron Microscopy (Jeol 6300F) was performed to examine the surface morphology of the microparticles. The lyophilized samples were carbon coated, imaged at 8 kV and viewed with magnifications of 100 and 300x.

2.5. Release kinetics and quantification

Release kinetics studies were conducted to determine the temporal release of PDGF from PDGF-SPLA microparticles. 200 mg of the PDGF-SPLA microparticles were placed in 15-ml conical tubes in the presence of 5 ml of PBS-AB (Phosphate-buffered solution containing 1% of antibiotics – Pen-Strep). All samples were placed in an orbital shaker and agitated at 120 rpm at 37ºC.

At pre-determined time points, 2,5 ml of the release supernatant (PBS-AB) were withdrawn and replaced with a similar volume of fresh PBS-AB. The supernatant was stored at −80ºC until analysis. Controls for the study included PBS-AB, SPLA microparticles (GF-free) and BSA-SPLA microparticles (table I), subjected to the same conditions as described for PDGF-SPLA microparticles.

Table I. Controls and samples used in the release experiments.

<table>
<thead>
<tr>
<th>Control/Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-AB</td>
</tr>
<tr>
<td>SPLA microparticles (GF free)</td>
</tr>
<tr>
<td>BSA-SPLA microparticles</td>
</tr>
<tr>
<td>PDGF-SPLA microparticles &gt;210 µm</td>
</tr>
</tbody>
</table>
The released PDGF was quantified using an Enzyme-Linked ImmunoSorbent Assay (ELISA) kit specific for human PDGF-BB (R&D Systems, MN, USA), by comparison to a standard PDGF-BB curve, according to the manufacturers’ instructions. All experiments were performed at least in duplicate.

2.6. Evaluation of the maintenance of the biological activity of released PDGF

When a molecule is incorporated inside a release system, it is vital that its biological activity is maintained throughout loading, incorporation and release. Thus, it was determined if PDGF incorporated into SPLA microparticles is still active after incorporation and release. To this end, it was used a culture of MC3T3-E1 cells.

However, before analyzing the biological activity of released PDGF, it was necessary to determine the minimal concentration of PDGF producing a mitogenic effect over MC3T3-E1 cells. Several concentrations of exogenous PDGF - that ranged from 0 to 50 ng/mL – were tested beforehand. Cells were seeded in 12-well tissue culture plates (Corning, USA) at a density of 12600 cells/cm². 24 hours after cell seeding, the culture medium was removed and replaced by fresh DMEM medium supplemented with the following concentrations of exogenous PDGF: 0, 2, 4, 10, 20 and 50 ng/mL. After 72 h, DNA levels were measured by the PicoGreen dsDNA quantification kit (Molecular Probes, USA). This is an ultra sensitive fluorescent nucleic acid stain for quantitating double stranded DNA (dsDNA) in solution that allows measuring cell proliferation.

At the end of the experiment, the culture medium was removed and the monolayer was washed with 10x Tris-EDTA buffer. 400 μL of 0.1 N NaOH was added to the wells to cause cells to release DNA into solution. An aliquot of the DNA suspension was removed and added to 10x Tris-EDTA buffer, to which was then added the PicoGreen reagent (previously prepared in 10xTE) in a 1:1 vol:vol, and fluorescence measured in a microplate reader at 485 and 535 nm excitation and
emission wavelengths, respectively. Lambda (λ) DNA was used as a standard. The results were obtained from three experiments.

After determining the minimal concentration of PDGF necessary to produce a mitogenic effect for MC3T3-E1 cells, the same cell density and experimental setting was used to test the samples and controls, as summarized in table II.

**Table II.** Controls and test conditions used for the evaluation of the biological activity of released PDGF.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Content of culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>DMEM</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + PBS-AB</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + supernatant of SPLA microparticles (GF free)</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + supernatant of BSA released from microparticles</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + exogenous BSA</td>
</tr>
<tr>
<td>Negative control</td>
<td>DMEM + BSA-SPLA microparticles</td>
</tr>
<tr>
<td>Positive control</td>
<td>DMEM + exogenous PDGF</td>
</tr>
<tr>
<td>Sample</td>
<td>DMEM + supernatant of PDGF released from microparticles</td>
</tr>
<tr>
<td>Sample</td>
<td>DMEM + PDGF-SPLA microparticles</td>
</tr>
</tbody>
</table>

After 3 and 6 days DNA content was quantified using the dsDNA PicoGreen kit, against a standard curve of known λ DNA concentrations. Results were obtained from three experiments.
2.7. Statistics

Results are expressed as mean ± standard deviation. Differences between experimental results were analyzed according to a Student t-test, with the threshold for statistical significance set at p<0.01.

3. RESULTS

3.1. Loading efficiency

The loading efficiency was determined by subtracting the amount of the incorporated molecule (either BSA or PDGF) to the initial amount available for incorporation. Table III presents the values of the loading efficiencies for both BSA and PDGF.

Table III. Loading efficiencies for BSA and PDGF-SPLA microparticles.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Loading efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>19±3%</td>
</tr>
<tr>
<td>PDGF</td>
<td>17±1%</td>
</tr>
</tbody>
</table>

As it can be observed from the results in table III, BSA incorporation is well correlated with the one for PDGF, confirming the effectiveness of BSA as a carrier/adjuvant for the incorporation of growth factors, as shown elsewhere. The loading efficiencies were around 17% of the initial amounts of molecules, which is a low value, even when compared with the loading efficiencies that these same microparticles displayed for other bioactive agents (for instance,
dexamethasone). However, works in literature incorporating PDGF make use of 3-D porous scaffolds\textsuperscript{22-24}, using different materials and processing methods, therefore they are inadequate for comparison purposes.

3.2. Release kinetics of PDGF-SPLA microparticles

The daily release profile for PDGF is shown in Figure 2. It can be observed that there is a burst release in the first 12 hours that decreases until 48 hours, after which a plateau is reached. This plateau in the release was maintained until the end of the release period – 8 weeks – where small amounts of PDGF were released each day from the matrix of the starch-based microparticles.

![Figure 2. Daily release profile of PDGF from PDGF-SPLA microparticles up to 8 weeks. A burst release is observed in the initial (12 h) release period, after which there is a slow release until the end of the studied 8-week period.](image-url)
3.3. Morphology – post release

In order to verify the integrity of these systems at the end of the release period (8 weeks), the morphology of the microparticles was compared with these same systems prior to the onset of the release study, as shown in images 3.A through 3.C.

![Figure 3. Morphology of pre and post release (8-week) PDGF-SPLA microparticles. A, individual microparticle before the onset of the release experiment; B, individual microparticle after the 8-week release period, evidencing massive degradation but still presenting a matrix structure; C overview of the morphology of 8-week release microparticles, where extensive degradation is visible.](image)

Drawing a comparison between particles before the release period (figure 3A) and particles after the 8-week release period (figure 3B), there is considerable degradation of the microparticles
after the 8-week release period. In figure 3B it is visible the extensive formation of pores at the surface of the microparticles, and in figure 3C it is possible to observe that the degradation extents to the population of particles. However, the shape of the microparticles is still maintained, with a high porosity matrix. This might indicate that the microparticles can still provide structural support after release of the incorporated agent, until new tissue fully replaces them.

### 3.4. Biological activity of released PDGF

The biological activity of the released PDGF from starch-based microparticles was assayed through the mitogenic effect of the bioactive agent over an osteoblastic cell line. Before evaluating the biological activity, it was necessary to determine the minimal effective concentration of PDGF that stimulates MC3T3-E1 cells to proliferate. Figure 4 reveals that almost all the concentrations tested – ranging from 2 to 50 ng/mL – are capable to stimulate MC3T3-E1 proliferation, except for the 50 ng/mL concentration (0 ng/ml vs 50 ng/ml, p= 0.0941). All other concentrations are able to stimulate the proliferation of cells significantly when compared to control conditions (0 ng/ml vs 2 ng/ml, p = 0.0003; 0 ng/ml vs 4 ng/ml, p < 0.0001; 0 ng/ml vs 10 ng/ml, p= 0.0001). The most striking effect is observed for the concentration of 4ng/mL, which showed up to a 4-fold stimulus in proliferation when compared to control (0 ng/ml).
Figure 4. Proliferative response of MC3T3-E1 cells to various concentrations of PDGF. All concentrations showed a stimulatory effect on cells, compared to control conditions. Except for the concentration value of 50 ng/mL, all conditions were statistically different from the control conditions.

Based on these results, 4 ng/mL was chosen as the concentration for testing of the biological activity PDGF released from starch-based microparticles.

The effect of released PDGF over MC3T3-E1 cells after 3 days was compared to control and exogenous PDGF. Figure 5 shows that when compared to control (cells fed with DMEM without supplementation) both released (RelPDGF) and exogenous PDGF (ExogPDGF) are able to significantly stimulate proliferation of MC3T3-E1 cells (Ctrl vs ExogPDGF, p = 0.003; Ctrl vs RelPDGF, p < 0.0001) after 3 days of culture. No differences are observed in DNA content between released and exogenous PDGF supplementation (RelPDGF vs ExogPDGF, p = 0.8526), confirming that biological activity of PDGF is maintained through incorporation and release.
Figure 5. Effect of released PDGF and exogenous PDGF over MC3T3-E1 cells at day 3. Both conditions – released (Rel PDGF) and exogenous PDGF (Exog PDGF) – are able to stimulate MC3T3-E1 cells to proliferate to values above control. There are no statistically significant differences between exogenous PDGF and released PDGF. Both conditions have been shown to statistically stimulate DNA content increase when compared with control (*).

The effect of all tested conditions over MC3T3-E1 cells was also evaluated after 3 and 6 days of culture, in order to establish the time frame and effect of PDGF over MC3T3-E1 cells. In figure 6 is displayed the DNA content of MC3T3-E1 cells for all conditions after 3 and 6 days of culture.

Figure 6. DNA content for 3 and 6 days demonstrating the effect of all conditions over MC3T3-E1 cells. At day 3 all conditions are statistically significant (*) when compared to control (Ctrl). At day 6 only SPLApart and PDGF-SPLApar DNA content are statistically significant when
compared to control conditions (**), to Exog PDGF (•) and Rel PDGF (•). Ctrl – Cells cultured without any supplement to culture medium; PBS – Phosphate Buffered Saline solution; Rel BSA – BSA released from starch-based microparticles; Exog BSA – BSA solution; SPLA-BSA – BSA-loaded SPLA microparticles; SPLApart – GF-free (unloaded) SPLA microparticles; Exog PDGF – PDGF solution at a concentration of 4 ng/ml; Rel PDGF – PDGF released from starch-based microparticles and supplemented at a concentration of 4 ng/ml; PDGF-SPLA part – PDGF-SPLA microparticles.

From figure 6 it is clear that the effect of PDGF over MC3T3-E1 cells is exerted in the first 3 days, after which all controls attain a similar level of DNA content comparable to PDGF-treated cells.

After 3 days of culture it is clear that conditions containing PDGF – exogenous (Exog PDGF), released (Rel PDGF) and PDGF-SPLA microparticles (PDGF-SPLApart) - are able to stimulate cell proliferation up to 4-fold compared to control. For this time period, only the above named conditions were able to significantly stimulate DNA content increase of MC3T3-E1 cells (Table IV).

Table IV. Results of the statistical analysis for pairwise comparisons at 3 days of culture.

<table>
<thead>
<tr>
<th>Pair wise comparison</th>
<th>p values</th>
<th>Statistical significance (99% confidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl vs PBS</td>
<td>0.0203</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs Rel BSA</td>
<td>0.0049</td>
<td>Yes (*)</td>
</tr>
<tr>
<td>Ctrl vs Exog BSA</td>
<td>0.0315</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs SPLA-BSA part</td>
<td>0.0007</td>
<td>Yes (*)</td>
</tr>
<tr>
<td>Ctrl vs SPLA part</td>
<td>0.0004</td>
<td>Yes (*)</td>
</tr>
<tr>
<td>Ctrl vs Exog PDGF</td>
<td>0.003</td>
<td>Yes (*)</td>
</tr>
<tr>
<td>Ctrl vs Rel PDGF</td>
<td>&lt;0.0001</td>
<td>Yes (*)</td>
</tr>
<tr>
<td>Ctrl vs PDGF-SPLA part</td>
<td>&lt;0.0013</td>
<td>Yes (*)</td>
</tr>
</tbody>
</table>
After 6 days of stimulation, all negative control conditions - with the exception of SPLA microparticles (SPLApart) - display similar DNA contents when compared with control cells (Ctrl). Similarly to SPLA microparticles, PDGF-SPLA microparticles (PDGF-SPLApart) constitute an exception to the trend of DNA content observed for all conditions. Only SPLA microparticles (SPLApart) and PDGF-SPLA microparticles’ (PDGF-SPLApart) samples are able to have statistically significant differences in DNA content when compared with non-supplemented cells (Ctrl) (***) and for positive controls (••), as confirmed by table V. This result confirms that PDGF mitogenic effect is exerted over MC3T3-E1 cells at the initial stages of the culture (3 days), after which non-supplemented cells reach similar DNA content.

**Table V.** Results of statistical analysis for pair wise comparisons at 6 days of culture.

<table>
<thead>
<tr>
<th>Pair wise comparison</th>
<th>p values</th>
<th>Statistical significance (99% confidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl vs PBS</td>
<td>0.1466</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs Rel BSA</td>
<td>0.1688</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs Exog BSA</td>
<td>0.0857</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs SPLA-BSA part</td>
<td>0.9731</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs SPLA part</td>
<td>0.0017</td>
<td>Yes (**)</td>
</tr>
<tr>
<td>Ctrl vs Exog PDGF</td>
<td>0.0746</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs Rel PDGF</td>
<td>0.7836</td>
<td>No</td>
</tr>
<tr>
<td>Ctrl vs PDGF-SPLA part</td>
<td>0.0022</td>
<td>Yes (**)</td>
</tr>
<tr>
<td>SPLA part vs Exog PDGF</td>
<td>0.0023</td>
<td>Yes (•)</td>
</tr>
<tr>
<td>SPLA part vs Rel PDGF</td>
<td>0.0017</td>
<td>Yes (•)</td>
</tr>
<tr>
<td>PDGF-SPLA part vs Exog PDGF</td>
<td>0.0027</td>
<td>Yes (•)</td>
</tr>
<tr>
<td>PDGF-SPLA part vs Rel PDGF</td>
<td>0.0021</td>
<td>Yes (•)</td>
</tr>
</tbody>
</table>
4. DISCUSSION

In this study we have evaluated the incorporation of PDGF in the matrix of starch-based microparticles and posterior release. Additionally, we have monitored the effect of released PDGF over cells of the osteoblastic lineage. The B isoform of PDGF was chosen because it has a greater mitogenic and chemotactic potential than PDGF-A, as well as a higher affinity to bone matrix $^4,^{13}$.

Comparing the loading efficiency with those of other systems described in the literature, the loading efficiency is rather low$^{2,25,26}$. However, in many of those systems the biological activity of the loaded growth factor is reduced during processing of the material, either by the action of organic solvents or by temperature inactivation. The system herein has the advantage that it is processed at room temperature and that only a residual amount of organic solvent is used, therefore minimizing the risk of GF inactivation. Incorporating PDGF into starch-based microparticles protects the GF and increases the half-life of the growth factor (GF) (which is very short when injected free), thereby compensating the rather low loading efficiency. Although only 17% of the initial amount of the growth factor is loaded (which will be the subject of further optimization), the amount of GF acting on the cells can be controlled by the amount of microparticles to be incorporated into the hybrid construct.

Biodegradable polymers normally display three separate release phases$^{27,28}$: 1) a burst or initial period of rapid diffusion of drug located close to the surface of the polymer, 2) a period of slow release, during which the polymer is gradually hydrolyzed in bulk and 3) a final release phase during which the polymer is solubilized in the aqueous environment. In the present work, the release profile observed for the tested time period comprises the first two of these phases, as evidenced by the 1) burst release in the first 12 hours and 2) a slow release, due to polymer degradation, as evidenced by the morphology at 8 weeks of release. While these characteristics are considered a drawback for many controlled release applications, in this particular case, for PDGF and bone applications, it might be advantageous. Hsieh & Graves showed that pulse release of PDGF enhances the formation of mineralized matrix in vitro, while continuous
application is inhibitory\textsuperscript{29}. PDGF is a potent mitogen and chemotactic agent for cells of the mesenchymal lineage, in which bone cells are included, and since proliferation and differentiation are seen as relatively uncoupled and sequential processes, it is considered to be a first phase effectors in bone\textsuperscript{13}. In this role it stimulates and recruits cells to the site in initial stages, after which it does not seem to play a leading role in cell fate and commitment, the latter attributed to other effectors, such as BMPs. At the light of these findings, the present release system can deliver PDGF in a burst to stimulate cells to migrate and expand in number to the defect site (\textit{in vivo}) or in the hybrid construct (\textit{in vitro}) and then maintain low delivery levels that will not impart the next phase of repair (maturation/differentiation). The release profile shows that after the burst release, starting at day 2, reduced levels of PDGF are released from the microparticles. When comparing with figure 4, where 4 ng/ml exert a significant effect over MC3T3-E1 cells, the fact that low amounts of the growth factor are released up to 8 weeks will not impart cell commitment to the osteoblastic lineage.

In the present study, when determining if the incorporation and release of PDGF in/from starch-based microparticles would have a deleterious effect on the biological activity of the GF, it was found that the mitogenic effect of released PDGF was comparable to the one of exogenous PDGF. BSA has been used extensively as an adjuvant for the loading of GFs\textsuperscript{2,18,30}, to protect them from the harsh organic environment encountered during processing steps. In the present study, BSA as an adjuvant protected PDGF from methylene chloride used in the production of the microparticles.

When comparing the effect of all conditions on the DNA content of MC3T3-E1 cells at day 3 of culture (figure 6) the following should be highlighted:

1. The presence of PDGF significantly stimulates an increase in the DNA content when compared with the control (cells in standard culture conditions, no supplements);

2. All other conditions seem to stimulate to some extent the DNA content of MC3T3-E1 cells. This effect however, is not as remarkable as the one observed for PDGF containing conditions;
3. Starch-based microparticles also seem to exert a positive effect over MC3T3-E1 cells, as evidenced by the result for SPLA microparticles (GF-free).

It could be hypothesized that degradation products from starch-based microparticles could have a stimulatory effect over the cells. We have shown before that 24 h leachables from SPLA microparticles are not toxic for MC3T3-E1 cells, as evidenced by the similar viability values as control conditions. At 6 days of culture, control cells reach similar DNA content compared to all other conditions, except for cells treated with PDGF-SPLA microparticles, which display a statistically significant increase in DNA content. Besides the stimulatory effect of PDGF, starch-based microparticles seem to stimulate DNA content increase. This hypothesis could be supported by data of MC3T3-E1 cells treated with SPLA microparticles (GF-free) at day 6 of culture, that also present a statistically significant increase in DNA content compared to control.

Cells treated with PDGF showed, besides enhanced proliferation, a fibroblast-like morphology (data not shown), in agreement to what was observed by Hsieh & Graves. However, the authors also report that this display of fibroblastic morphology does not impart the formation of a mineralized matrix, since cultures treated with PDGF for 3 days and then transferred to new plastic dishes exhibited a 70% increase in mineralized nodule area compared to controls. These results predict that multiple, brief exposures to PDGF would enhance bone formation in vivo, while prolonged exposure to PDGF, which is likely to occur in chronic inflammation, would inhibit differentiated osteoblast function and limit bone regeneration. This being the case, the use of this specific growth factor should be limited to small time periods, up to 3 days. As so, the system herein presented might be very useful in stimulating proliferation and expansion of cell number for TE applications.

Platelets are known reservoirs of several growth factors, and among them, PDGF is predominant. Growth factors within the platelet-rich plasma (PRP) are believed to mediate normal bone healing and regeneration. In this way, several works have shown that platelet extracts can be applied to stimulate bone cells to proliferate. Bearing this in mind, rather than using a
purified growth factor (with a high cost), an effective strategy could be the one to incorporate platelet extract obtained from the patients’ own platelets into the above proposed carriers. This strategy could further increase the effectiveness of the system in TE applications.

In bone tissue engineering strategies, the combination of materials possessing adequate properties - such as biocompatibility, osteoconductivity, physical and chemical properties – and site-specific cells has the potential to become a very successful means of repair. If coupling with these properties the ability to entrap and release stimulatory molecules such as growth factors with a defined temporal profile, the advantages of such system would be several-fold.

Current Tissue Engineering strategies involve scaffold materials and cells. Herein, in a new strategy, we propose the use of starch-based microparticles to act simultaneously as (i) biodegradable scaffold materials, (ii) carriers for cells and (iii) carriers/vehicles for the incorporation and release of bioactive agents, such as growth factors.

5. CONCLUSIONS

The ability of starch-based microparticles to incorporate, release and maintain the biological activity of Platelet-Derived Growth Factor was described. The release of bioactive agent occurred at greater amounts in initial time periods – up to three days - when it was shown to stimulate the most MC3T3-E1 cell proliferation. From the results, MC3T3-E1 cells were markedly stimulated to proliferate upon supplementation with PDGF, both exogenous and released from starch-based microparticles, confirming the mitogenic effect of this growth factor upon cells of the osteoblastic lineage and the maintenance of its biological activity after incorporation and release. After the release period was observed extensive degradation of the microparticles, but they were still able to maintain their 3-D structure.
Combined with previous properties described for these starch-based microparticle systems – biocompatibility, cell adhesion properties and biological activity – the ability to release active growth factors sustains the potential of these systems to be used for bone tissue engineering applications.
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General Conclusions

The goal of the present thesis was to develop new microparticle systems for drug delivery applications and for innovative Tissue Engineering strategies. For that we have used natural origin, biodegradable starch-based polymers, and a glass material, Bioactive Glass 45S5, to produce particle systems that could be further evaluated for biomedical applications.

The overall work was divided into the following items:

- To develop novel micron size particles using natural origin materials and combinations with other groups of materials (such as ceramics);
- To characterize the developed systems in a physicochemical manner;
- To study the interaction of the developed materials with cell cultures, in particular populations from an eventual target tissue;
- To determine the ability of the developed systems to be used for drug delivery applications.

The previous items are part of a strategy where the developed systems can be used simultaneously as carriers for cells in dynamic culture conditions (bioreactors) while at the same time acting as release systems for biologically active agents, to further enhance cell functions.
1. DEVELOPMENT OF PARTICULATE SYSTEMS

In order to produce particulate systems, several starch-based materials were tested, using several synthesis methodologies, mostly based on the creation of emulsions. We here discuss the two selected materials: Paselli 2, a chemically modified potato starch and a blend of cornstarch with polylactic acid (SPLA).

For the production of Paselli 2 particles, it is necessary to crosslink starch chains in order to obtain a three dimensional (3-D) structure to form a particle. Trisodium trimetaphosphate (TSTP) was shown to be more effective as a crosslinker than epichlorohydrin in crosslinking of starch-chains. Additionally, the later presents the problem of extremely high toxicity. This aspect could be potentially deleterious for further cell culture studies, in the event of incomplete removal of un-reacted epichlorohydrin.

The emulsion method used for the production of soluble starch particles (Paselli 2) was found difficult to control, mainly the formation of a stable emulsion. This method makes use of paraffin oil, whose removal was also found to be an additional drawback. For Paselli 2 other methodologies were tested, but none of them was found to be as effective as the one referred above. The reinforcement with 30% of Bioactive Glass 45S5 was found to produce composite microparticles with superior properties in terms of a more spherical morphology, even surface, and additional stability at room temperature.

When attempting the formation of particles using a blend of cornstarch with polylactic acid (SPLA), a simple emulsion method allowed obtaining particles with a well-defined range of sizes (0-350 µm). The use of a crosslinker (TSTP) was tested, but did not show significantly different results. The method was found to be simple to perform and to produce a high yield of particles (high ratio of produced particles to raw material).

The production of starch-based particles aimed additionally to obtain particles that could be used for dynamic culture conditions. The underlying idea is to have a system that can be used as substrate for cell culture in dynamic conditions, to stimulate cell proliferation and differentiation
while producing a hybrid construct that can be used for tissue engineering. The dynamic bioreactor to be used was the NASA-approved bioreactor, the HARV (High Aspect Ratio Vessel). Studies by Ducheyne and co-workers have shown that these systems can be used for the formation and differentiation of three-dimensional rat marrow stromal cell culture on microcarriers for tissue engineering applications\(^1\)\(^-\)\(^3\). The ideal carrier system to be used with this bioreactor needs, however, to possess particular characteristics, such as a density equal or close to the density of the fluid to be used in the bioreactor culture system. At the end of the reaction for production of SPLA particles, a group of particles could be observed to float at the top of the reaction medium. However, when this group of particles was analyzed, it was found to be mostly composed of the PLA component of the raw material. Subsequently, several attempts have been made to obtain SPLA particles with lesser density, by the following strategies:

- Reducing the amount of raw material, but maintaining all other parameters (volume of solvents and stirring speed). The hypothesis was this could lead to the production of less dense particles. This strategy showed no significant differences in the resulting particles compared to the "standard" synthesis methodology.

- Increasing the stirring speed to reduce the size of the produced particles. It was found that particles with sizes around 20-70 \( \mu \)m were able to circulate within a defined trajectory without colliding with the outer wall of the vessel for an extended period of time. However, these sizes were not adequate for cell culture purposes.

- Incorporating an agent that would render the particles less dense. Attempts were made to incorporate an agent used in industrial applications as a spraying aerosol for paints, Microlithe® glass that has extremely low density. However this feature of the agent presented additional problems to its incorporation, as in the emulsion this material would not be kept in the same phase of the starch-based material.

None of the proposed strategies was able to increase the ratio of lighter to denser particles.
In summary, using a wide range of starch-based materials and methodologies, we were able to select two starch-based materials and respective methodologies for the production of starch-based particles, both polymer and composite with Bioactive Glass 45S5, based on parameters such as morphology, size distribution and methodology.

2. PHYSICOCHEMICAL CHARACTERIZATION OF STARCH-BASED PARTICLES

The characterization of the developed starch-based particles involved several aspects, including size distribution, morphology, hydration degree, degradation, bioactivity and cytotoxicity.

Size distribution

Size distribution within each batch of Pa2 particles was mostly possible to be determined only for composite particles and it was found to be consistently reproducible. For Pa2 polymer particles, freeze-drying was effective in removing any aqueous content, but in the presence of minimal moisture/humidity the particles would aggregate, rendering the sieving process for size separation almost impossible.

For SPLA polymer and composite particles, size distribution was found not to be influenced by the presence of Bioactive Glass 45S5, as sizes were found to be within the same range and with the same distribution.

For both starch-based materials, particles were found to be in the micron size range, reason for their designation throughout this thesis is as microparticles.
Morphology

In the case of Pa2 microparticles, both polymer and composite were shown to possess spherical morphology and the surface of composite microparticles appeared more homogenous than that of Pa2 polymer microparticles. The latter's rough surface seemed to suggest each particle was formed by the union of smaller microparticles. The presence of Bioactive Glass seems to have a stabilizing effect on the structure of the microparticles, in the sense of conferring them additional stability at both room temperature and 37°C.

In the case of SPLA (polymer and composite) microparticles, there are no differences in terms of morphology between polymer and composite microparticles. Microparticles are spheroid and at their surface rougher areas alternate with smoother areas, which are attributed to the starch phase and PLA phase, respectively. The distribution of these areas could not be controlled during production, and this variability in distribution is probably caused by the fact that there is the formation of a PLA core, with the starch phase distributed randomly in the particle. This feature will obviously cause differences in the behavior of the microparticles in other studies.

Hydration and degradation

All materials were found to have a high hydration degree, with values for swelling reaching 300%. For other starch-based materials, it was found that the synthetic component of the blend influences the water uptake, with hydrophobic materials reducing the diffusion of water and therefore reducing the water uptake. The high water uptake values indicate that if these systems are used for controlled release applications, the initial drug release stage will be controlled by diffusion of the molecules through channels created by water penetration. Regarding the degradation rate, this was found to be more pronounced in the first two weeks, where the materials loose around 20% of their initial weight. This result is in agreement with what was observed for other starch-based materials.
While for other starch-based materials the addition of a ceramic phase (hydroxylapatite) increased the weight loss\(^9\), in the present case the bioactive glass incorporated within the matrix formed by the polymeric phase slowed down the degradation of the material. Bioactive glasses contain SiO\(_2\) as a network former, which results in slow and incomplete resorption, and this might explain the lower weight loss observed for composite starch-based microparticles compared with the polymeric ones.

**In vitro bioactivity**

The materials were also studied for their *in vitro* bioactivity, or the ability to allow the formation of a calcium-phosphate layer at their surface when immersed in a simulated body fluid, which seems to be correlated with the ability of a material to bond to bone\(^{10-13}\).

Polymers in general are not bioactive, so it was surprising to find that Pa2 polymer microparticles were able to nucleate calcium and phosphate at their surface. However, due to the presence of phosphate in the crosslinked structure of the particles it was not possible to determine the Ca-P ratio at the surface. The reinforcement of starch-based microparticles with 30% of Bioactive Glass 45S5 in the structure of starch-based microparticles renders them bioactive, as evidenced by formation of a Ca-P layer at the surface of both types of composite microparticles (SPLA/BG and Pa2/BG). Analysis of the deposited layer showed that it is partially crystalline and seems to possess a Ca-P ratio similar to the one of apatite from osseous tissue. This might be an indication of the behavior of these systems in terms of integration and stimulation of an osseous defect.

**Cytotoxicity**

Microparticles were also evaluated for their eventual cytotoxicity using the typical mouse fibroblast cell line, L929. All types of microparticles were found to be non-cytotoxic for the cell line.
For Paselli 2 microparticles, this behavior is dependent on the washing process. Extensive washing is required in order to reduce possibility of cytotoxicity from these microparticles due to residual solvents, mainly from the oleic phase.

3. INTERACTION WITH CELLS FROM THE OSTEOBLASTIC LINEAGE

Cells from the osteoblastic lineage were used to evaluate the response of cells in terms of adhesion, proliferation and maintenance of the phenotype/differentiation when cultured at surface of different starch-based microparticles.

In a first approach we used soluble starch (Pa2) polymer and composite (with BG 45S5) microparticles and rat bone marrow cells (RBMCs). The cells were isolated and cultured at the surface of the microparticles up to 14 days, and although there is some residual fluorescence from the material – which is much more evident for the composite microparticles, due to the presence of BG 45S5 – nevertheless there is expression of osteopontin by rat bone marrow cells. Additional data coming from other osteoblastic markers, such as osteocalcin, Runx-2 and evidences of matrix deposition, could confirm that these cells have in fact differentiated into osteoblasts.

Difficulties within this work arose from the fact that Pa2 polymer microparticles lost their integrity in culture conditions somewhat around day 14 in culture. These particles are not able to maintain their structure throughout the culture period, so we decided not to continue further with these systems in terms of cell culture. Composite microparticles, however, do maintain their integrity for the duration of the culture (disregarding the effective changes caused by degradation), which only highlights the beneficial effects of the addition of BG 45S5. Additional difficulties were related with (i) the difficulty of performing the static culture, since there was the possibility of losing some particles whenever the culture medium was changed, (ii) light microscopy was impossible to perform since the particles were opaque and (iii) cell seeding techniques needed further optimization, as there was the feel that the drop method (where the cell suspension was dropped...
over the ensemble of particles laying at the bottom of the culture well) didn’t allow an even distribution of cells at the surface of the particles.

The use of cell lines allows one to have a more defined and accurate system to evaluate the effect of the materials over cell population. Based on this principle, a mouse calvaria-derived cell line -MC3T3-E1 pre-osteoblastic cells - was used for cell culture at the surface of starch-based microparticles.

As a first step, the adhesion of MC3T3-E1 cells to starch-based microparticles should be evaluated. For this purpose, we used Green Fluorescence Protein (GFP)-modified MC3T3-E1 cells, which allow detection under a fluorescence microscope. Although starch-based microparticles are not ideally observed in a light microscope, it was possible to observe only some fluorescence from cells adhered to the surface starch-based microparticles. In an attempt to increase cell adhesion to starch-based microparticles, the following approaches were taken under consideration:

- Surface modification by chemical methods (such as by a potassium permanganate treatment);
- Pre-treatment of starch-based microparticles by immersion into FBS/DMEM;
- Optimization of the cell seeding methodology.

For other starch-based materials, surface modification treatments have shown to be effective in enhancing cell adhesion to the surface of the modified materials\textsuperscript{14}. A treatment based on potassium permanganate, which produces starch macroradicals that can react with the oxygen from the air, enriching the surface of the material in oxygen by the formation of peroxide and carboxylic groups\textsuperscript{14}, was performed. This treatment did not show statistically significant improvement of MC3T3-E1 cell adhesion at the surface of starch-based microparticles. This can be explained by the fact that SPLA and Paselli2 microparticles are rich in OH groups, and introduction of more oxygen is only significant in starch-based materials with lower oxygen
content, such as SEVA-C\textsuperscript{14}. Although improved hydrophilicity is regarded as responsible for an increase in cell adhesion and proliferation\textsuperscript{15-17}, a balance between hydrophilic and hydrophobic domains must exist, and too many hydrophilic domains might impart protein adsorption to the surface of the materials and consequently cell adhesion.

In order to further evaluate which was the optimal condition for cell adhesion, a suspension of MC3T3-E1 cells was added to a pre-determined amount of starch-based microparticles in one of the following conditions: (i) dry, without hydration prior to cell seeding, (ii) hydrated in 100% FBS prior to cell seeding and (iii) hydrated in complete DMEM prior to cell seeding. The results showed decreasing cell adhesion in the following order DMEM > FBS > no hydration. In some samples there was no difference between microparticles hydrated in DMEM and FBS. This result was the basis for choosing DMEM for hydration of the microparticles prior to cell seeding experiments.

Another approach to stimulate cell adhesion involved the optimization of the cell seeding methodology. For the studies with RBMC and Pa2-based microparticles, we used a drop method for cell seeding, that is, in a pre-determined volume of particles, a cell suspension in a reduced volume of culture medium was carefully added to the population of microparticles. Despite its demonstrated effectiveness in porous starch-based scaffolds\textsuperscript{18}, this method does not show the same effectiveness for starch-based microparticles. The surface area of the microparticles is greatly enhanced compared with other systems (in the size range of 210-350 µm, average diameter size is 280 µm, the surface area is around 880 µm), but the spherical shape of the microparticles does not keep cells in a restricted area. Also, the distribution of cells at the surface of the microparticles is determined by the position of the particle, because the surface facing up will have more adhered cells than surfaces facing down or sides. For this reason, the seeding method by immersion of microparticles into a cell suspension might be more effective than the drop cell seeding method. Additionally, cells will preferentially attach to the surface of tissue culture well. This problem was obviated before with the use of non-adherent tissue culture plates.

In an attempt to overcome the problems discussed above, a modified cell seeding technique was developed, involving cell seeding using conical tubes instead of tissue culture wells and low
speed centrifugation. The advantage of this method consisted of the closer proximity of cells and particles and the possibility for cells to adhere to all the surface of a microparticle. To further increase the probability of cell adhesion to the surface of starch-based microparticles, a low-speed, short-term centrifugation was performed. Additionally, we used a system that prevented particle loss during medium exchange at the different time points. Cell strainers, regularly used to separate individualized cells from cell clumps, were used to prevent loss of microparticles during medium exchange, thus keeping the samples in reproducible conditions.

One unexplored strategy to evaluate cell adhesion and function at the surface of starch-based microparticles is the formation of pellets (by compression of samples) that would allow for an easier handling and testing of the samples. However, it was our feeling that the system would not mimic the conditions where the microparticles could be used, for instance in a dynamic culture situation.

Pre-osteoblastic cells were seeded at the surface of starch-based microparticles, and they were shown to adhere to the surface of the SPLA and SPLA/BG microparticles around 40% and 60%, of the number of cells adhering to TCPS, respectively. A similar behavior was observed for osteoblastic cells in PDLLA scaffolds, where cells adhered in higher percentage to composite scaffolds containing bioactive glass than to polymer ones, but in both cases at lower percentages than the one observed for control conditions (TCPS)\textsuperscript{19}. Similarly, cell proliferation showed to be reduced when compared with standard tissue culture conditions, and this was also described for PDLLA scaffolds\textsuperscript{19}. This result can be due to several reasons: (i) differences in surface properties within the same particle caused differential cell attachment; (ii) a density effect - the formation of 3-D aggregates causes cells within the inner areas of the aggregate to have less access to nutrients and also reduced area for proliferation and (iii) the degradation of the material removes potential adhesion sites for cells. In general, cell behavior and interaction with a biomaterial surface is dependent on properties such as topography, surface charges and chemistry\textsuperscript{20,21}. In order to clarify the results obtained for cell adhesion, an extensive analysis of the surface properties of the materials would be very useful, namely the oxygen:carbon ratio. However, since these materials
are shaped as microparticles, determination of the contact angle and XPS studies are extremely
difficult to perform.

Nevertheless, when evaluating the expression of osteoblastic markers, such as osteopontin,
osteocalcin and Runx-2, starch-based microparticles, especially composite, have shown to
enhance the expression of the above-referred markers. Concomitantly, the analysis of calcium
deposition (as a marker of matrix deposition by cells) showed significant enhancement both by
polymer and composite microparticles when compared to standard culture conditions. These
effects might have their origin on the spatial arrangement cells within the aggregate. Additionally,
and in particular for composite microparticles, the presence of Bioactive Glass 45S5 and its
dissolution products enhances cell functions such as enhanced phenotypic markers expression,
as described in the literature\textsuperscript{22}.

In summary, although cell adhesion and proliferation at the surface of starch-based
microparticles were lower than the ones observed for standard 2-D tissue culture conditions, the
cells cultured at the surface of the materials were shown to be committed to the osteoblastic
phenotype and to have enhanced expression of osteoblastic markers and calcium deposition, in
particular for composite microparticles. This effect is mostly mediated by the presence of Bioactive
Glass 45S5, which has been shown before to stimulate osteoblastic gene expression.

4. INCORPORATION AND RELEASE OF BIOACTIVE AGENTS

SPLA microparticles were selected for testing of loading and release bioactive molecules. The
choice of this material was based on the following criteria:

- The method for producing these microparticles was simpler when compared with the
  production methodology for Pa2 microparticles;

- The yield of the method for these systems was higher than for Pa2 microparticles;
- SPLA microparticles are more stable at room temperature when hydrated and maintain their structure longer at 37°C than Pa2 microparticles;
- Washing steps are faster and reduced when compared with Pa2 microparticles.

The first molecules to be studied were corticosteroids, specifically dexamethasone. The choice of this bioactive molecule was based on the use of dexamethasone as a differentiation agent for marrow cells to commit to the osteoblastic lineage. The aim was to entrap dexamethasone within the matrix of starch-based microparticles and to culture cells at the surface of these polymer systems. Upon dexamethasone release, cells would be stimulated to differentiate into the osteoblastic lineage, as the released dexamethasone could be readily accessible for cell stimulation. To this end, dexamethasone and two other corticosteroids—16α-methylprednisolone and 16α-methylprednisolone acetate—were individually loaded by incorporation into the matrix of SPLA microparticles. The last two corticosteroids were studied for comparison purposes, as the basic structure of the three molecules is the same (methylprednisolone), with differences only in the side groups. The differences observed in the release profile are a consequence of the presence of the side groups and the spatial arrangement of the molecule, i.e., the “bulkiness” of the molecule.

Dexamethasone was released with an initial burst followed by a slow, steady release, which is typical for biodegradable polymers, and does not allow any control over the release system. In the present work the activity of dexamethasone over cells cultured at the surface of the loaded microparticles was not evaluated. Additionally, no specific bioassay for released DEX biological activity assessment was performed. However, released dexamethasone analyzed by HPLC was unaltered by incorporation and release, as concluded by the comparison of the peaks of released DEX and unloaded DEX.

This approach was, however, further exploited in the study that aimed to assess if starch-based (SPLA) microparticles could serve as carriers for growth factors known to be effective in the osseous tissue regeneration process. PDGF is known to be a potent mitogen for cells from several
lineages, among them cells from the osteoblastic lineage\textsuperscript{25,26}. To this end, Platelet-Derived Growth Factor (PDGF) was entrapped and its release profile determined, as well as the maintenance of its bioactivity after these two processes. As stated before, this last aspect should also have been evaluated for dexamethasone loaded in and released from SPLA microparticles.

PDGF incorporation showed a low efficiency. However, it is noteworthy to highlight that the released growth factor was able to maintain its bioactivity through loading and release, as shown by the potent mitogenic effect observed over cultures of MC3T3-E1 cells. Similarly to what was above stated for DEX loaded microparticles, it would have been desirable that PDGF-loaded microparticles were used for cell culture of MC3T3-E1 cells at their surface, in order to determine if the release of the growth factor would stimulate MC3T3-E1 cells proliferation. This would allow evaluating if these systems could perform as carriers for growth factors and cells, as part of hybrid constructs for TE purposes. Nevertheless, starch-based microparticles were shown to be adequate vehicles for the incorporation and release of biologically active molecules for at least two groups of agents: corticosteroids and growth factors.
FINAL REMARKS

Related to each of the aims, the following points summarize the work carried under the scope of this thesis.

DEVELOPMENT OF NEW MICROPARTICLES…

• It was possible to develop micron size particles, both polymeric and composite, using several starch-based materials and ceramic materials. The methodologies employed have shown to be adequate to produce these systems, which allowed, to some extent, tailor the properties of the microparticles, namely their size distribution and morphology.

• The characterization of the developed systems has shown that these are biodegradable and hydrophilic, as seen by their degradation over time and water uptake profile, respectively. These systems are simultaneously bioactive, as evidenced by the formation of a Ca-P layer at their surface and non-cytotoxic in vitro. The combination of these properties seems adequate for tissue engineering applications.

…FOR DRUG DELIVERY APPLICATIONS…

• Starch-based microparticles were able to entrap and release bioactive agents in a sustained fashion. Dexamethasone and two other corticosteroids were entrapped at dramatic high loadings, and were released with an initial burst, followed by a steady state release up to 30 days. Platelet-Derived Growth Factor (PDGF) was loaded with lower efficiency compared to the corticosteroids, however it was released in a sustained fashion and with a profile adequate for tissue engineering
applications. Its bioactivity (as denoted by its mitogenic effect) is maintained during loading and release. As conclusion, these systems present an outstanding potential for drug delivery applications.

...AND INNOVATIVE TISSUE ENGINEERING STRATEGIES

- Cells from the osteoblastic lineage (namely pre-osteoblastic cells and rat bone marrow cells) are able to adhere to the surface of the developed starch-based microparticles, to proliferate and to form stable 3-D aggregates during the culture period. When analyzed for their ability to maintain their osteoblastic phenotype/commit to the osteoblastic lineage, both MC3T3-E1 and rat bone marrow cells, respectively, where found to express osteoblastic markers during the culture period and to mineralize in culture.

When gathering all the information from each of the aims, it can be drawn that the developed starch-based microparticles, although further characterization needs to be performed, present potential to be used as carriers for bioactive molecules, much more than the potential to be used as carriers for dynamic cell culture conditions. Further understanding of intrinsic properties of these developed systems (in terms of surface properties) would allow optimization of the systems in order to enhance their potential to be used as carriers for cells in dynamic culture conditions.

In the proposed strategy, the micron size systems aim to fill different roles, such as: support, carriers for cells and as system for the controlled release of bioactive agents. Within this strategy, we aimed to develop starch-based microparticles that could fulfill the above roles. The results show that we were capable of developing starch-based microparticles whose characterization found to be biodegradable, non-cytotoxic, bioactive, able to support cell adhesion and the maintenance of the osteoblastic phenotype, and simultaneously serve as carriers for the
incorporation and release of biologically active agents such as anti-inflammatory agents and growth factors.

In summary, the results confirm the potential of the developed starch-based micron size systems for the above proposed strategy. This confirms that the milestones for this work were achieved.

FUTURE WORK...

Future course of this work would have to contemplate the following aspects:

- **Optimize the developed starch-based microparticles to be used in dynamic cell culture conditions, namely using the HARV bioreactor.**

Preliminary work within this aim showed that starch-based microparticles can circulate in a defined trajectory within the vessel, and this ability is mainly dependent on their size. Larger particles circulate closer to the other wall and hit it in a shorter time (figure 1) when compared with smaller size particles.

**Figure 1.** Example of the behavior of starch-based microparticles (in this case, Paselli2-BG 45S5) within the size range 210-350 μm. The speed of rotation of the vessel was 8 rpm. Frames were taken sequentially within a minute when the vessel reached the same position.
Also, for aggregates formed between microparticles and MC3T3-E1 cells prior to placement in the HARV were found to maintain their integrity and to be able to circulate with a defined trajectory without hitting the outer wall. This ability was found to be under control of the rotational speed of the vessel.

The aggregates were maintained for 3 weeks within the vessel without loss of integrity or significant changes in the trajectory. However, cell proliferation seems to be impaired in this system, since the result of an MTT revealed absence of cell proliferation.

Preliminary data obtained from RT-PCR showed that cells cultured under these specific dynamic conditions display a similar array of gene expression as cells cultured in static conditions (figure 2).

![Figure 2](image_url)

**Figure 2.** RT-PCR data for gene expression by MC3T3-E1 cells cultured at the surface of SPLA/BG 45S5 microparticles in static (S) culture and HARV (H) dynamic culture conditions. Marker – 100 bp marker; GADPH, housekeeping gene; OSP, osteopontin; OCN, osteocalcin; COL I, collagen Iα and Runx-2, transcription factor.
- Characterize these systems extensively in terms of surface properties (chemistry, functional groups, phase distribution, among others) to clarify cell adhesion and behavior of cells cultured at the surface of the microparticles.

- Study the ability of - similarly to polymer SPLA microparticles – composite SPLA/BG 45S5 microparticles to entrap and release PDGF, dexamethasone, eventually other bioactive agents.

- Culture cells at the surface of PDGF-loaded and Dexamethasone-loaded microparticles, to evaluate the feasibility of the proposed concept – to have systems that can be simultaneously used as support materials, substrates for cells and carriers for the delivery of bioactive molecules.

The data obtained from the experiments performed under the scope of this thesis seem to point to a positive result under this aim, namely when starch-based microparticles loaded with PDGF were supplemented to culture medium fed to MC3T3-E1 cells and these were shown to proliferate significantly when compared with cells fed with DMEM supplemented with exogenous (non-loaded) PDGF.
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