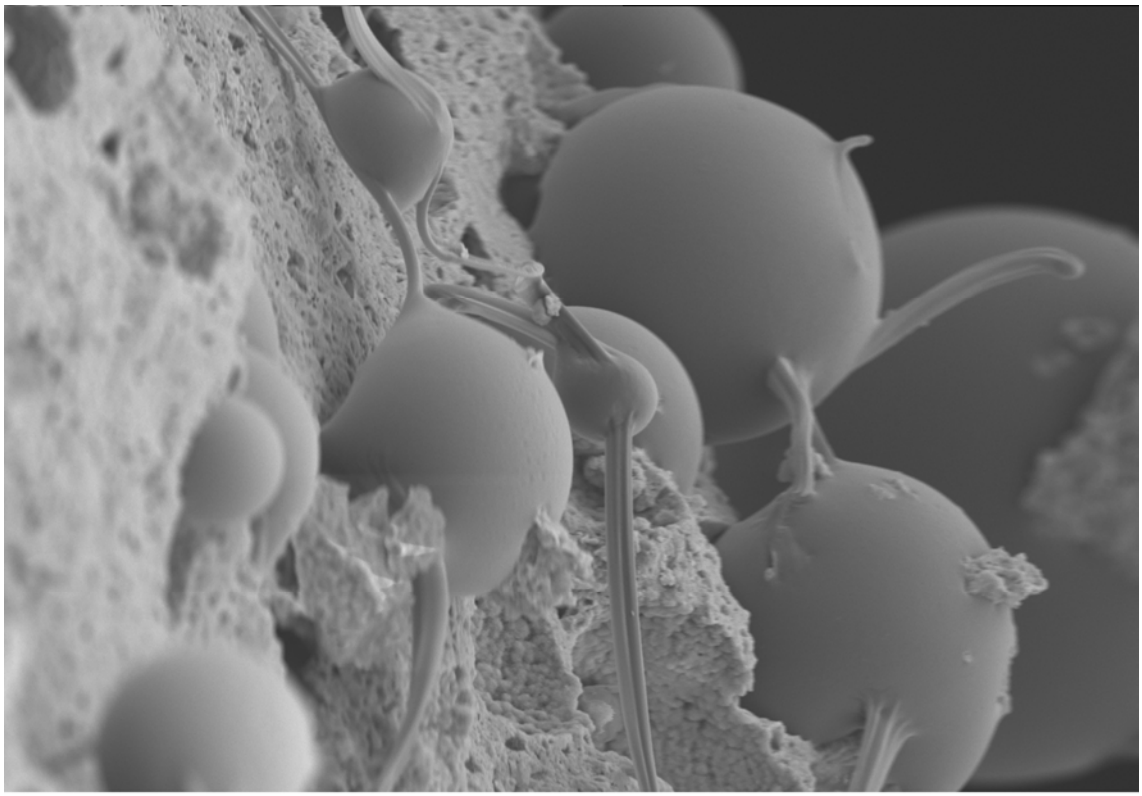
# Development of biodegradable calcium phosphate cement for bone tissue engineering



## Colofon

Thesis Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, with summary in Dutch.

Development of biodegradable calcium phosphate cement for bone tissue engineering
Woutherus Johannes Elisabeth Maria Habraken, Nijmegen, 2008


ISBN 978-90-9022990-4

| | |
|---|---|
| Cover Design & lay-out | Wouter Habraken |
| Printing | Print Partners Ipskamp BV, Enschede, The Netherlands |

# Development of biodegradable calcium phosphate cement for bone tissue engineering

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus, prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op woensdag 4 juni 2008,
om 13.30 uur precies

door

Woutherus Johannes Elisabeth Maria Habraken
geboren op 4 december 1979
te Tiburg

Promotor: Prof. dr. J.A. Jansen

Copromotor: Dr. J.G.C. Wolke

Manuscriptcommissie:
Prof. dr. J.C.M. van Hest (voorzitter)
Dr. A.H.M.S.M. van Kuppevelt
Prof. dr. W. Hennink, Universiteit Utrecht

Paranimfen:
Sebastiaan Canoy
Ruud van Deursen

# Development of biodegradable calcium phosphate cement for bone tissue engineering

An academic essay in the field of Medical Sciences

Doctoral thesis

to obtain the degree of doctor
from Radboud University Nijmegen
by the authority of the rector magnificus, prof. dr. S.C.J.J. Kortmann,
according to the decision of the Counsil of Deans
to be defended public on Wednesday 4th of June 2008,
at precisely 13.30 hours

by

Woutherus Johannes Elisabeth Maria Habraken
born in Tilburg, the Netherlands
on December 4, 1979

Supervisor: Prof. dr. J.A. Jansen

Co-supervisor: Dr. J.G.C. Wolke

Manuscript committee:
Prof. dr. J.C.M. van Hest (chairman)
Dr. A.H.M.S.M. van Kuppevelt
Prof. dr. W. Hennink, University of Utrecht

Ushers:
Sebastiaan Canoy
Ruud van Deursen

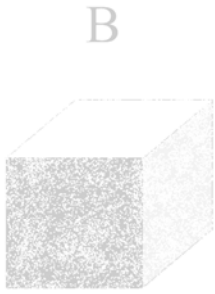
# Contents

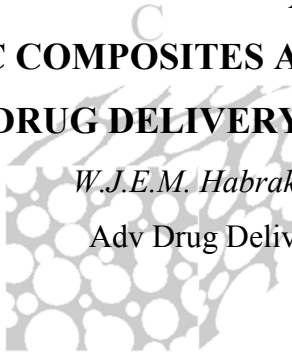

| | | |
|---|---|---|
| CHAPTER 1 | General Introduction | 9 |
| CHAPTER 2 | Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics | 39 |
| CHAPTER 3 | PLGA microsphere/calcium phosphate cement composites for tissue engineering: *in vitro* release and degradation characteristics | 57 |
| CHAPTER 4 | Introduction of gelatin microspheres into an injectable calcium phosphate cement | 75 |
| CHAPTER 5 | Porcine gelatin microsphere/calcium phosphate cement composites: *in vitro* degradation and protein release | 99 |
| CHAPTER 6 | *In vitro* growth factor release from injectable calcium phosphate cement containing gelatin microspheres | 119 |
| CHAPTER 7 | Introduction of enzymatically degradable poly(trimethylene carbonate) microspheres into an injectable calcium phosphate cement | 135 |
| CHAPTER 8 | *In vivo* degradability of calcium phosphate cement with incorporated biodegradable microspheres | 155 |
| CHAPTER 9 | Closing remarks and future perspectives | 175 |
| CHAPTER 10 | Summary and address to the aims | 181 |
| CHAPTER 11 | Samenvatting and realisatie van de doelstellingen | 187 |
| | Dankwoord | 193 |
| | Curriculum Vitae | 195 |
| | List of Publications | 196 |
| | Color Figures | 198 |

# CHAPTER 1

## GENERAL INTRODUCTION





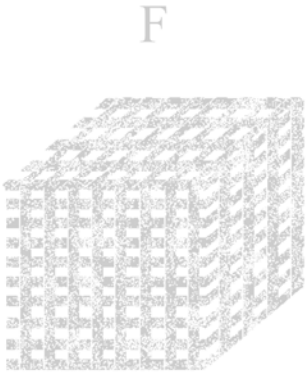
*Adapted from:*

**CERAMIC COMPOSITES AS MATRICES AND SCAFFOLDS FOR DRUG DELIVERY IN TISSUE ENGINEERING**

*W.J.E.M. Habraken, J.G.C. Wolke, J.A. Jansen*

Adv Drug Deliv Rev. 59(4-5) (2007) 234-245



## 1. Introduction

Ceramics can be described as "solid compounds that are formed by the application of heat, and sometimes heat and pressure, comprising at least one metal and a nonmetallic elemental solid or a nonmetal, a combination of at least two nonmetallic elemental solids, or a combination of at least two nonmetallic elemental solids and a nonmetal"[1]. Ceramics comprised of calcium phosphates, silica, alumina, zirconia and titanium dioxide are nowadays used for various medical applications due to their positive interactions with human tissues. Examples can be found in the field of dentistry[2] where calcium phosphate and calcium hydroxide-based materials are used as endodontic filling materials and metal-ceramic alloys are applied for crowns. Also in the field of orthopedics[3-6] (invertebral disks, joints) and plastic surgery[7] (cranial defects) ceramics are frequently employed. Characteristics of these ceramic materials are often a high mechanical strength, a good body-response and a low or non-existing biodegradability.

Due to the slow degradability, for tissue engineering purposes these materials don't appear to be very attractive. However, in the field of bone tissue engineering most scaffolds are ceramic or ceramic-derivatives. Especially hydroxyapatite-based calcium phosphate compounds[8,9] and bioactive glass[10] are regarded as high-potential scaffolds due to their osteoconductive properties. To improve the biodegradability of these implants, porosity is introduced[11] which also gives rise to bone ingrowth, though pore sizes must be large enough. This porosity is sometimes also called macroporosity while the ceramic implants can have a micro or nanoporosity of their own. A parameter that is important here for tissue engineered bone constructs is the so-called "interconnectivity" of the pores. It implies that the pores introduced into these scaffolds overlap each other, and instead of separate cavities, lanes of interconnected pores are generated. Figure 1 gives an overview of typical ceramic 3D-structures.

Next to the scaffold material, in bone tissue engineering cells and growth factors are also introduced to speed-up tissue ingrowth. In most ceramic scaffolds, however, difficulties arise when cells are added, most probably due to a limited supply of nutrition at the inside of the implant or less than optimal cell-cell interactions.

Growth factors like bone morphogenic protein-2 (BMP-2), transforming growth factor (TGF-β), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are commonly introduced into these scaffolds due to their osteoinductive properties and vascularization[12-14]. Though ceramics like hydroxyapatite are known for their positive interaction with bone morphogenic proteins (BMP's), high amounts of these growth-factors are added in *in vivo* studies to guarantee a positive tissue response. A reason for this phenomenon is that ceramics can bind strongly to the growth-factors/proteins, or give a release pattern that is less than optimal. This increases the clinical relevant amount high above normal values inside the human body and increases the cost of a single implant substantially, therefore decreasing a possible use of the material. Alternatives for the use of growth factors are for example the use of ceramics with osteoinductive properties, as described for biphasic

calcium phosphate (BCP)[15], a composite of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP).

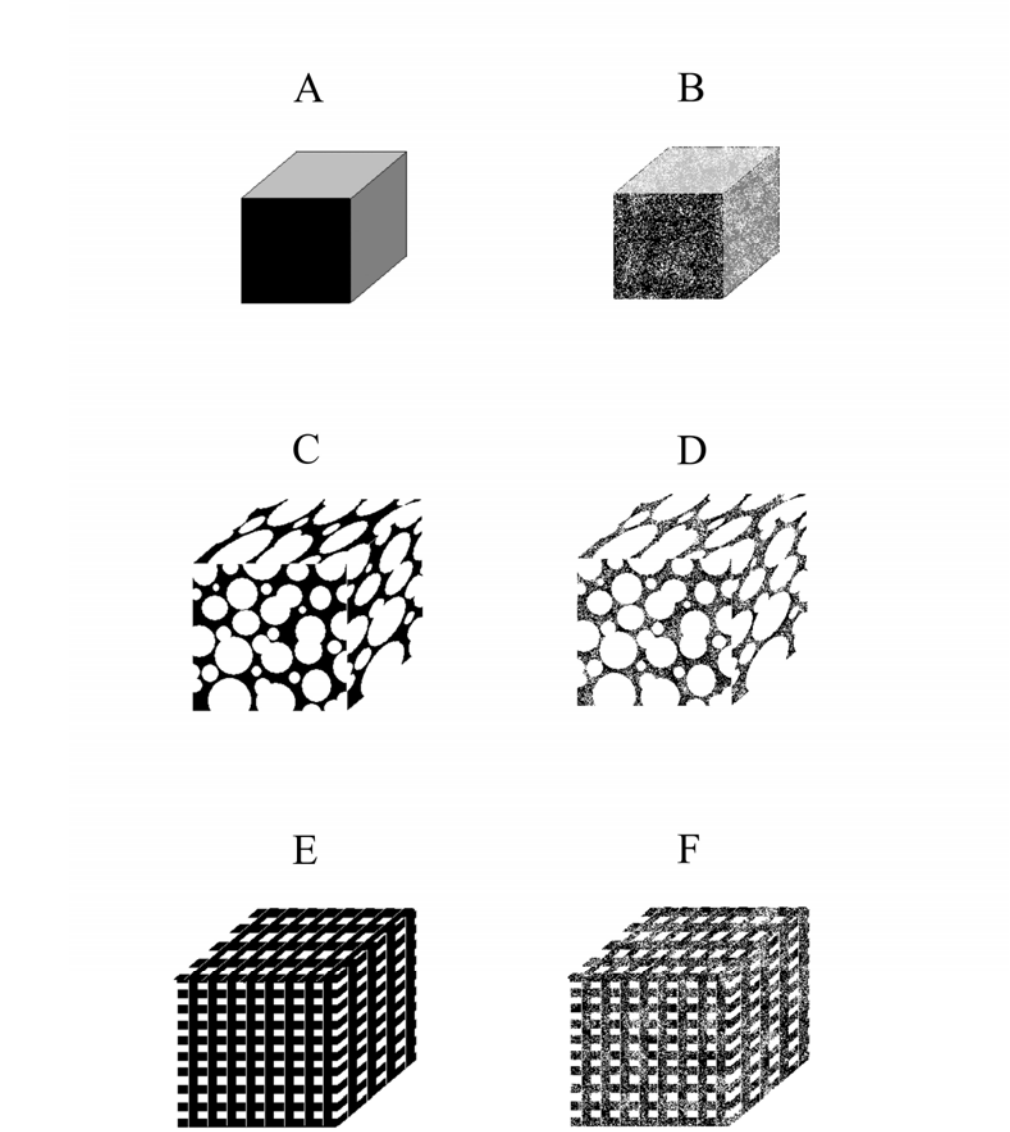



**Figure 1.** Ceramic 3d-structures; A = non-porous, B = microporous, C = macroporous (spherical), D = macroporous (spherical) + micropores, E = macroporous (3d-printing), F = macroporous (3d-printing) + micropores

Another possibility is the use of other materials like (bio)polymers that are physically or chemically mixed with a ceramic compound. Examples of polymers that are commonly used in combination with ceramics are the polylactic acid-based polymers[16] and biopolymers like chitosan[17] and collagen[18]. Nowadays, these composite materials of organic/inorganic origin are studied extensively because they can combine the tailored degradability and high release efficiencies of the polymer with the osteoconductivity and delayed/sustained release characteristics of the ceramic material. Next to that, also mechanical and physical properties of the polymer can compromise the brittleness of the ceramic material or give better handling properties to calcium phosphate (CaP) cement.

Overall, the characteristics of an ideal ceramic composite for bone tissue engineering should comply to the following parameters:

1. A biodegradability that is enabling bone remodeling
2. A macroporosity that enables cell ingrowth into the composite
3. Mechanical stability/ease of handling
4. Osteoconductivity to guide bone around/inside the implant
5. Carrier for growth factors/cells

In the next paragraphs, ceramics and ceramic/organic composites are summarized that are studied in the last decades and their use for bone tissue engineering and drug release will be discussed according to the ideal scaffold characteristics.

## 2. Ceramic composites

As mentioned in the introduction, ceramic composites are nowadays commonly used in a wide variety of medical applications. In this section, ceramic composites without the addition of organic components are reviewed. An overview of some commercially available ceramics, cements and bioactive glass is given in Table 1. An index of abbreviations of CaP-compounds with corresponding chemical formula and Ca/P-ratio is given in Table 2.

### *2.1. Calcium phosphate ceramics*

Due to their similarity to human bone, calcium phosphate ceramics were and still are very popular implant materials for diverse clinical applications since the last 30 years. Different types of CaP-ceramics are available, though they can be classified as either hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), biphasic calcium phosphate (BCP), amorphous calcium phosphate (ACP), carbonated apatite (CA) or calcium deficient HA (CDHA).

The use of these materials for tissue engineering purposes is still explored. Most researchers are aware that the low resorbability of sintered CaP-ceramics, and in particular HA or CA forms a problem. The use of highly porous implants induces bone formation inside the implant and increases degradation. Complete resorption in most cases is very difficult due to the crystalline architecture. Porosity in these ceramics can be introduced by different techniques where in most cases a structure of interconnected (spherical) pores is produced. This can be accomplished by leaching and sintering out of salt crystals or polymeric microparticles/mold after which the ceramic remains. Mastrogiacomo et al.[19] for example used a cellulose matrix to serve as mold for a HA-paste, after which the cellulose was removed by pyrolysis and after sintering an open porous HA matrix remained. A similar technique is based on rapid prototyping[20]. Here, a HA scaffold with controllable shape/size and porosity is produced after casting the calcium phosphate onto a negative mold, produced by rapid prototyping, and burning out the mold.

**Table 1.** Commercially available ceramics, cements and bioactive glass

| Ceramic/ cement | Consistancy | Endproduct | Setting time | Com.Strength (MPa) | Porosity |
|---|---|---|---|---|---|
| Biosorb | β-TCP | Granules<br>Ca/P = 1.49-1.51 | - | 15-150 MPa | 5-45%<br>macro+micro |
| Calcibon | Calcium deficient CA | granules | - | 4-7 MPa | micro+macro |
| Ceraform | 65% HA<br>35% TCP | granules | - | 50MPa | <10% |
| Chronos | β-TCP | granules | - | 7.5 MPa | 60-80%<br>macro |
| Endobon | Bovine HA | granules | - | 2.5-16 MPa | high |
| Interpore | HA (coral) | granules | - | high | 35% |
| Pro-Osteon | HA (coral) | granules | - | 2-4 Mpa | 65% |
| Triosite | 60% HA<br>40% β-TCP | granules,<br>Ca/P = 1.6 | - | >10 MPa | 70%<br>macro+micro |
| Vitoss | β-TCP | granules | - | low | 88-92% macro |
| Biobon (α-BSM) | ACP<br>DCPD | Poorly cristalline HA, Ca/P = 1.45 | 15-20min (37°C) | 12 MPa | 50-60%<br>micro |
| Biopex | 75% α-TCP<br>18% TTCP<br>5% DCPD<br>2% HA | HA | 8min | ± 80MPa | micro |
| Bone Source | TTCP<br>DCPD | HA, Ca/P =1.67 | 10-15 min (37°C) | 36 MPa | micro |
| Calcibon | 61% α-TCP<br>26% DCPA<br>10% $CaCO_3$<br>3% pHA | Calcium deficient CA<br>Ca/P = 1.53 | 2-4min (37°C) | 60-70MPa | 44%<br>micro |
| Cementek | α-TCP<br>TTCP<br>$Ca(OH)_2$ | HA, Ca/P = 1.64 | 40 min | 20MPa | 50%<br>micro |
| Chronos inject | 42% β-TCP<br>21% MCPM<br>31% β-TCP granules<br>5% $MgHPO_4*3H_2O$ | Brushite (DCPD) | 12 min | 3 MPa | micro |
| Mimix | α-TCP<br>TTCP<br>citric acid | HA | 2-4 min (37°C) | 22 MPa | micro |
| Norian-SRS | MCPM<br>α-TCP<br>$CaCO_3$ | Dahlite (CA)<br>Ca/P = 1.67 | 12 hr | 28-55 MPa | very low |
| Bioglass 45S5 | 45% $SiO_2$<br>24.5% $Na_2O$<br>24.5% CaO<br>6% $P_2O_5$ | Different shapes/films | - | - | low |

Also surfactant (foaming methods) can be used to produce spherical porosity inside the CaP-ceramic. Almiral et al.[21] used $H_2O_2$ as a foaming agent. In this study $O_2$-gas was produced by reaction of the hydrogen peroxide, which formed the foam and porosity inside the ceramic. Some ceramics are nature derived and have a distinct porous structure of their own, like the coralline hydroxyapatite derived ceramics (Interpore®, Pro-Osteon®) and the bovine derived ceramics (Endobon®). These materials are very similar in structure as human bone; however, a drawback of these commercially available

ceramics is that the porosity/mechanical strength has a fixed shape and value and therefore cannot be tailored for a specific purpose.

Next to the macroporosity that enables cell ingrowth, in some cases the CaP-ceramic also has a micro/nanoporosity. Because of the limited pore size, this porosity seems not of interest for tissue engineering, however, the microporosity improves fluid flow though the ceramic, thereby providing nutrition for cells inside the scaffold.

**Table 2.** Abbreviations of CaP-compounds with corresponding chemical formula and Ca/P-ratio

| Abbreviation | Name | Formula | Ca/P-ratio |
|---|---|---|---|
| ACP | Amorphous calcium phosphate | - | 1.25<x<1.55 |
| BCP | Biphasic calcium phosphate | $Ca_3(PO_4)_2 + Ca_{10}(PO_4)_6(OH)_2$ | 1.50<x<1.67 |
| CA | Carbonated apatite, dahlite | $Ca_5(PO_4,CO_3)_3$ | 1.67 |
| CDHA | Calcium deficient hydroxyapatite | $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ | 1.50<x<1.67 |
| DCPA | Dicalcium phosphate anhydrous, Monetite | $CaHPO_4$ | 1.00 |
| DCPD | Dicalcium phosphate dihydrate, Brushite | $CaHPO_4*2H_2O$ | 1.00 |
| HA | Hydroxyapatite | $Ca_{10}(PO_4)_6(OH)_2$ | 1.67 |
| MCPM | Monocalcium phosphate monohydrate | $Ca(H_2PO_4)_2*H_2O$ | 0.50 |
| OCP | Octacalcium phosphate | $Ca_8H_2(PO_4)_6*5H_2O$ | 1.33 |
| pHA | Precipitated hydroxyapatite | $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ | 1.50<x<1.67 |
| α-TCP | α-Tricalcium phosphate, Whitlockite | $\alpha$-$Ca_3(PO_4)_2$ | 1.50 |
| β-TCP | β-Tricalcium phosphate, Whitlockite | $\beta$-$Ca_3(PO_4)_2$ | 1.50 |
| TTCP | Tetracalcium phosphate, Hilgenstockite | $CaO*Ca_3(PO_4)_2$ | 2.00 |

Because of the positive influence of ceramics on cell differentiation/proliferation, it is not surprising that bone forming cells are often introduced into these ceramics to speed-up tissue ingrowth. The surface of sintered ceramics is chemically stable and therefore a good substrate for seeding cells. Adding cells to tissue engineered scaffolds is often called a necessity, conversely, even inside the 100% interconnected macroporous scaffolds there is high cell morbidity and cell survival is restricted to the surface layer. Kruyt et al.[22] applied a multiple-condition model consisting of polyacetal cassettes in which different CaP-ceramics were placed consisting of BCP, β-TCP and HA. In these scaffolds bone marrow stromal cells (BMSC's) were loaded and the effect on bone ingrowth was investigated. The effect of added BMSC's was visible especially with BCP and β-TCP, which overall exhibited better bone ingrowth than HA. Furthermore, the BMSC's showed a more pronounced effect in an ectopic site when compared to a bony environment, which was explained by a better vascularization and therefore lower cell morbidity at the ectopic site.

Adding osteo-inductive growth factors/drugs to CaP ceramics is very convenient. Due to the high affinity of the ceramics for drugs/proteins, one can load these scaffolds just by absorption of a growth-factor containing fluid onto the ceramic. As discussed earlier, the high affinity of CaP ceramics for proteins/growth factors can also result in retention of a high percentage of this material. The release pattern of most loaded ceramics seems to consist of an initial burst release (elution of not bound protein) followed by a specific release dependent on material/drug. Stallman et al.[23] added gentamycin to different calcium phosphate granules and observed a similar release pattern for all

granules with only a very low sustained release. Release efficiencies reached to 30-62% after 17 days, indicating that the ceramic retained a substantial amount of the gentamycin. Guicheux et al.[24] added 5µg of human growth hormone onto a macroporous BCP ceramic and observed a rapid release within the first 48h, followed by a slow sustained release exponentially decreasing until day 11. Though this was a less than optimal release pattern, 100% of the human growth hormone was released and showed structural integrity and biological activity. To achieve a more sustained release, Kimakhe et al.[25] applied dynamic compaction to a CDHA scaffold loaded with polymyxin B. The release pattern observed was indeed more sustained following Higushi's law[13]; however, the dynamic compaction decreased the biological activity of the drug.

Degradation of growth factors in time also can occur as Ziegler et al.[26] observed with rxBMP-4, rh-bFGF and rhVEGF loaded α-TCP ceramics, neutralized glass ceramics, a glass ceramic/PLGA composite and dehydrated human bone. Here, different release curves and efficiencies were obtained depending on the type of growth factor and comparing the ceramics. Looking at the materials, dehydrated human bone showed the weakest loading capacity, releasing 70-95% of the loaded drug within three days. A comparison between the growth factors showed a looser binding of VEGF to the α-TCP ceramic, and an upregulation of BMP-4 after 3 days with all synthetic scaffolds. Surprising was that within most groups molecular weight of the growth factor decreased dramatically after 3-5 days in release medium, especially with BMP-4 and bFGF. This result indicates that degradation of protein structures can occur when incorporated inside ceramics/release media for a longer period. The upregulation of BMP-4 after 3 days therefore was also discussed to be the result of degradation. Bioactivity of the released fractions was not recorded, though it would be interesting to see if these degraded protein structures still have a bioactivity.

Next to the introduction of growth factors, another possibility is to use a scaffold that has an osteoinductive potential of itself. Research by many authors show that calcium phosphate materials, especially biphasic calcium phosphate (BCP) and HA have some osteoinductivy of themselves when implanted in muscle tissue[15,27]. About the process of osteoinductivity there is still a lot of speculation, though the presence of macro and microporosity seems to be essential[28]. Whether ceramics alone can induce enough bone to make growth factors/cells obsolete is debatable, though it would make the final application much easier in use and lower in cost.

### *2.2. Calcium phosphate cements*

Next to calcium phosphate ceramics, calcium phosphate cements are of interest for bone tissue engineering purposes. Calcium phosphate cements consist of a powder phase of calcium and/or phosphate salts that together with an aqueous phase react at room/body temperature and form a calcium phosphate precipitate that sets by the entanglement of crystals. Due to the low processing temperature, they don't comply with the original definition of ceramics, though the powder phase contains sintered CaP-material (α-TCP, HA). The main difference between cements when compared to

ceramics is the injectability and in-situ hardening of the cement which makes it a material that is easy to handle from a clinical point of view[29, 30]. A ceramic has to be shaped to the defect site during operation which makes it very labor intensive, and often a less than optimal tissue-to-implant contact is obtained. Since CaP-cements were invented by Chow et al.[31], a lot of different formulations have been investigated with variable success. In general there are two types of CaP cement: apatite cements that set into HA, CA or CDHA and brushite cements that have DCPD (brushite) as end product. Without going too far into the literature about the two cement types one can say that microporous apatite cement is slow degrading and has a good mechanical stability. Brushite cement overall has a lower mechanical strength but a faster biodegradability than the apatite cement. Both types of cement can be applied for tissue engineering purposes. Comparable to CaP-ceramics, introduction of macroporosity into the material is a necessity. Unlike most sintered CaP-ceramics, CaP-cements also have a high nano/sub-micron-sized porosity. This porosity enables fluid flow inside the composite; however, it is too small for tissue ingrowth. Macroporosity can be introduced using different methods, rendering a highly porous system. Examples are the use of water-soluble crystals that after injection just dissolve, or degradable polymer microspheres that will be discussed in a following chapter. Xu et al.[32] used mannitol crystals that were mixed with the CaP cement powder, after which the set cement was immersed in water. These particles generate macroporosity but should be present in a high percentage to assure interconnectivity. Another method is to introduce a foaming agent inside the material that generates gas-bubbles in the cement. Del Real et al.[33, 34] used sodium bicarbonate to generate these bubbles inside an apatite cement. The pores that were formed were irregularly shaped and quite large. As a consequence, compressive strength decreased to very low values when compared to the original cement. *In vivo* resorption was significantly accelerated, though the cement was not injectable anymore. Recently, Ginebra et al.[35] developed an injectable macroporous CaP-cement with the use of albumin as porogen. These cements have a more regular and interconnected macroporosity and still retain or even increase their injectability. Due to the smaller pore size generated, the compressive strength is slightly higher than the cement produced by the gas bubble method.

Adding cells to cement scaffolds is difficult due to the chemically unstable environment and the mixing process. Link et al.[36] observed that a commercially available apatite cement (Calcibon®) showed *in vitro* cytotoxicity though *in vivo* an excellent biocompatibility is observed. The cytotoxic reaction here is caused by dissolution of one of the cement components that releases acidic products. In *in vivo* studies, the cement experiences a more dynamical flow that neutralizes the acid.

Growth factors/drugs can be added to the cement just by adding the growth factor/drug to the liquid hardener, thereby distributing it equally through the cement. As the setting process can be influenced by the addition of proteins to the liquid phase, the addition of high amounts delays cement setting and therefore forms difficulties for clinical use. Growth factors like BMP-2/TGF-beta that are commonly used for tissue engineering purposes are only added in small amounts, and therefore the influence of these growth-factors on cement setting can be regarded as insignificant. Carrier proteins, like bovine

serum albumin (BSA), are present in much larger amounts and therefore have a higher ability to influence the setting reaction. On the other hand, Blom et al.[37] added rhTGF-β in BSA carrier solution (2 mg/ml) to an apatite-based cement, but observed no increase in setting time. The addition of albumin to cement even can be beneficial for release properties. A study of Ruhé et al.[38] showed a higher *in vitro* release of rhBMP-2 out of an albumin containing macroporous scaffold, though *in vivo* no difference was observed. The albumin here serves as a buffer between the cement and the growth factor, providing a weaker bond.

Release characteristics from calcium phosphate cement are well-studied and in many cases consist of a cement dissolution initial stage (during cement setting), followed by a diffusion-dependent release that is influenced by material characteristics[13]. When compared to ceramics, the occurrence of a high burst release is very rare, mostly due to the different administration of the drug. Release of drug due to the (*in vivo*) resorption of the material is much more complicated though has be to taken into account with high resorbable porous or brushite cements. Also the type of drug influences the release rates when these drugs are incorporated inside the cement. Stallman et al.[23, 39] determined the release properties of gentamycin and human lactoferrin 1-11 (hLF1-11) from different commercially available microporous CaP cements in different studies but under similar conditions. The release of gentamycin after 7 days was between 36-85%, while with hLF-11 the total release only reached to 1.9-33.8%, indicating a much higher retention. The high spread in release percentage here is caused by the difference in cement type which was divided into low and high retention cements. Next to that, the release curves of hLF-11 almost showed no initial burst release, while with the gentamycin loaded cements this occurred in most of the formulations. A concentration dependency on the drug release % is also possible, though the protein absorbing ability of CaP-cements is very high and release patterns in most cases look similar. *In vivo* studies using TGF-β and BMP-2 inside macroporous/microporous scaffolds show positive bone response on the introduction of these growth factors, proving that the released material is still active[12, 14, 40-43]. Seeherman et al.[40] observed accelerated healing in a nonhuman primate fibular osteotomy model using a single percutaneous injection of rhBMP-2 in macroporous CaP-cement. For this model a concentration of 1.5 mg/ml (0.75 μg/injection) was optimal which was fully released within 6 weeks. Also Ruhé et al.[41, 42] observed increased bone healing and osteoinductive properties of rhBMP-2 loaded macroporous CaP-cement scaffolds when implanted in cranial defects and at ectopic sites in rabbits. The amount of growth factor used here was 10μg/implant, where *in vivo* release studies show a 60% release of rhBMP-2 after 4 weeks[38]. Blom et al.[43] used TGF-β-loaded microporous CaP-cement to fill bone defects in the skull of rats. They applied 10 or 20 ng TGF-β to the cement and observed an increase in bone healing when compared to the non-loaded scaffolds, although the effect was minimal. Because of the high costs of the growth-factor and looking at the wide range of amounts of growth factor applied in *in vivo* research, it is important to use as little as possible regarding the final application. Drug release efficiencies therefore

should be optimized, though they are strongly dependent on the type of growth factor / carrier and implant site.

### *2.3. Bioactive glass*

Bioactive glass is the name of a range of glass compositions that have the ability to bind to bone and other tissues as was discovered by Hench in 1969[44]. It is produced like conventional glass in which the basic components are $SiO_2$, $Na_2O$, CaO and $P_2O_5$, and is commercially available as Bioglass®. More recently, bioactive glasses are also made using the sol-gel route which results in finer porous structures with a higher bioactivity than the conventional glasses with the same composition[45]. Each component has its own contribution to the bioactivity of the bioactive glass, but CaO or $Na_2O$ can be substituted without significantly affecting bone bonding. Even $P_2O_5$-free glasses are bioactive, where it was first assumed that this was a required component. The bone bonding ability is based on the chemical reactivity of the bioactive glass in which silicon bonds are broken and finally a CaP-rich layer is deposited on top of the glass which crystallizes to hydroxycarbonate apatite (HCA). Mechanical properties of bioactive glass are not optimal, and therefore other ceramic components are sometimes added to the bioactive glass for reinforcement. These materials are also referred to as glass-ceramic composites[46, 47].

For tissue engineering purposes, next to the bioactivity, the bioactive glass should be degradable and porous. The methods for preparing porous bioactive glass are similar to porous CaP ceramics. Yuan et al.[48] used a $H_2O_2$-foaming method to obtain a macroporous bioactive glass with pore sizes ranging from 100-600μm. Chen et al.[49] was able to prepare a 86-93% porous bioactive glass by use of a polyurethane template which was immersed in a glass slurry. The glass adhered to the polymer, excess was removed, and the polymer was burned out after which the scaffold was sintered. Kaufmann et al.[50] used PEG-glycol particles of different sizes and amounts to retrieve a porous bioactive glass substrate after sintering. Degradability of bioactive glass is mainly a dissolution based procedure. Dissolution rates can be influenced by the particle size, glass type and type of medium[51, 52]. However, overall the biodegradation of these materials is very low.

Drug loading of bioactive glasses is possible using the sol-gel method. Santos et al.[53] added trypsin inhibitor to the bioactive glass by absorbing it to the sols in a protein content of 0.6, 1.6 and 3.3% by weight. To measure the release of the trypsin inhibitor, samples were crushed into granules and release was measured in buffer solution. The cumulative release after 4 weeks was 21.2-43.4%. The release pattern was characterized by an initial stage of slow release, followed by a second stage in which the release had a linear relationship against the square root of time. Domingues et al.[54] introduced tetracycline and tetracyline/β-cyclodextrin into a bioactive glass and observed an initial burst of 12%, followed by a sustained release over 80 days and a total release of 22-25%. Also, growth factors were added to a neutralized bioactive glass ceramic[26]. Here an initial release of 10% was seen, followed by a delayed boost between day 3 and 8, depending on the type of growth factor. Efficiencies of 80%

were obtained, though only the growth factor released during the initial burst showed structural stability.

## 3. Ceramic/polymer composites

Next to the studies in which the tissue engineered scaffold only exists of the ceramic material, nowadays there is a lot of research ongoing on so-called ceramic/polymer scaffolds. From the previous three paragraphs it can be concluded that ceramic scaffolds alone can be a suitable implant material for bone tissue engineering while macro/microporosity can be introduced, they can form a suitable scaffold for cells and serve as a delivery vehicle of osteoinductive drugs. On the other hand there are some problems that are very difficult to solve with the ceramic scaffold alone, like poor degradability of the material and mechanical properties, i.e. tensile strength and brittleness. The addition of biodegradable polymers can improve the degradability of the ceramics/cements and alter their mechanical/ physical properties. Also, drug release profiles can be altered because there is a wide range of different polymers that can be selected, which show different degradation rates and mechanisms. In the next paragraphs especially two types of ceramic/polymer composites are reviewed: cement with added polymers and ceramic particles into a (porous) polymer carrier. An overview of the various polymers discussed in this section is given in Table 3.

**Table 3.** Summary of polymers used in combination with ceramics, categorized in groups

| Group | Polymers |
|---|---|
| Polylactic/polyglycolic acid | polylactid acid (PLA, PLLA, PDLLA), polyglycolic acid (PGA), poly(lactic-*co*-glycolic acid) (PLGA), poly-ε-caprolactone (PCL) |
| Proteins | collagen, gelatin, fibrin, casein, peptides |
| Carbohydrates | chitin, chitosan, cellulose, starch, alginate, hyaluronan, hydroxymethylpropylcellulose (HPMC), amylopectin |
| Other polymers | poly(propylene fumarate) (PPF), polycarbonate, polyalkanoates, poly(1,8-octanediol-citrate) (POC), poly(ethylene glycol) (PEG), poly (ethylene imine) (PEI), poly(ethylene oxide) PEO, prolypropylene (PP), nylon, aramide, poly(allylamine hydrochloride) (PAH) |

### *3.1. Polylactic/polyglycolic acid-based polymer/ceramic composites*

Polylactic/polyglycolic acid derived polymers are well studied and many types have already been investigated. Members of this ester-backbone polymer group are polylactic acid (PLA, PLLA), polyglycolic acid (PGA), poly-(d/l-lactic-*co*-glycolic) acid (PLGA) and poly-*ε*-caprolactone (PCL). The polylactic/glycolic acid group is interesting due to its biocompatibility and degradation properties that can be tailored by changing different parameters, for example the molecular weight, tacticity and L/G-ratio in the case of PLGA. The degradation mechanism is a hydrolysis reaction in which the glycolic acid residue degrades faster than the polylactic acid while the cleavage site of PLA is sterically hindered. Overall the degradation rate is in the following order: PGA > PLGA > PLA > PCL. Because of the tailored degradability, a scaffold can be formulated in which the degradation

properties are adapted to the final application. Most polylactic/ceramic scaffolds therefore exploit these degradation properties by using the polymer as the scaffold material.

Ceramic particles are added to the polymer to make it (more) osteoconductive and/or to increase mechanical properties. These scaffolds can be either porous or dense of structure using bioactive glass, HA, ACP and BCP as ceramic component in a PLLA, PLGA or PCL matrix. For tissue engineering purposes, a porous structure is preferred, though dense scaffolds are also frequently investigated. Navarro et al.[55] made a glass-ceramic particle/PLA scaffold by homogeneous mixing of the glass-ceramic into a PLA solution after which $H_2O$-solvable NaCl particles were added to create macroporosity in the scaffold. Mechanical properties of the PLA increased after adding up to 40% of glass-ceramic particles, still compressive strength was very low. Conversely, Niemela et al.[56] observed a decrease in bending strength/modulus as well as shear, compression and torsion strength when bioactive glass was added to a poly-L,DL-lactide (70/30) carrier by twin-screw extrusion. Despite the unfavorable mechanical properties, bioactive glass increases the bioactivity of the polymer carrier, as was observed by Yao et al.[57] where the addition of bioactive glass particles promoted osteoblastic differentiation of marrow stromal cells.

Zhang et al.[58] prepared highly porous composites (85.1-95.6%) of HA and PLLA/PLGA by solid-liquid phase separation and solvent sublimation of a polymer/HAP mixture in dioxane/water. These composites showed reinforcement when compared to pure PLLA. Shikinami et al.[59] even made reinforced dense composites of sintered HA inside a PLLA matrix and obtained mechanical properties equivalent/higher than cortical bone. Degradation of these materials, however, is slow, and complete resorption will take many years[60].

A porous composite of PCL with 10/20% CaP particles was investigated by Mondrinos et al.[61]. Compared to pure PCL, the compressive strength of these porous scaffolds produced by a 3d printing method, increased dramatically. Human embryonic palatal mesenchymal cells were added to these composites, which showed an increased proliferation in the 20% CaP composites. Next to the usual ceramic particles/polymer matrix, other ceramic/polylactic composites have also been formulated[62, 63]. For example, Ambrioso et al.[63] prepared a macroporous scaffolds by sintering composite CaP/PLGA microspheres where the CaP-phase was trapped inside the microspheres.

The addition of cells to porous polylactic acid-based composites normally does not provide difficulties, especially when slow degrading PCL or PLLA is used. Faster degrading polymers, like low molecular weight PLGA or PLA, can give a cytotoxic reaction due to the production of acidic degradation products. This is not only visible when cells are implanted but also *in vivo* studies with these polymer/ceramic composites without the addition of cells can show a cytotoxic reaction. Ignatius et al.[64] observed a strong inflammatory response after 24 months in sheep when composites of PLA and α-TCP or a glass ceramic were implanted. This inflammatory response occurred concomitantly with a strong reduction of the PLA and mechanical strength.

Polylactic/polyglycolic acid-ceramic implants can also be used as scaffolds for drug release where the drug can be incorporated into the polymer[65] or absorbed onto the composite[26]. Soriano et al.[65] added gentamycin to calcium phosphate/PLA blends and release curves showed a burst release followed by a slower sustained release, typical for PLA polymers. After coating the implants with PLA, the burst release was reduced and a sustained release over a period of 10 weeks was observed. As discussed in the ceramics paragraph, growth factors were also added to these composites[26].

Polylactic/glycolic acid-CaP cement composites can be formulated by the introduction of PLA/PGA microspheres. While CaP-cements are processed at room temperature, polymer microspheres can be added as a porogen forming pores after *in vivo* / *in vitro* degradation and as a possible carrier of growth factors. Drug release from degradable PLLA/PLGA microspheres is studied extensively and has proven to be useful for the release of proteins/drugs. Preparation methods for these microspheres are diverse and microspheres of different sizes and morphology can be prepared using a either a double-emulsion method[66] or spray drying[67].

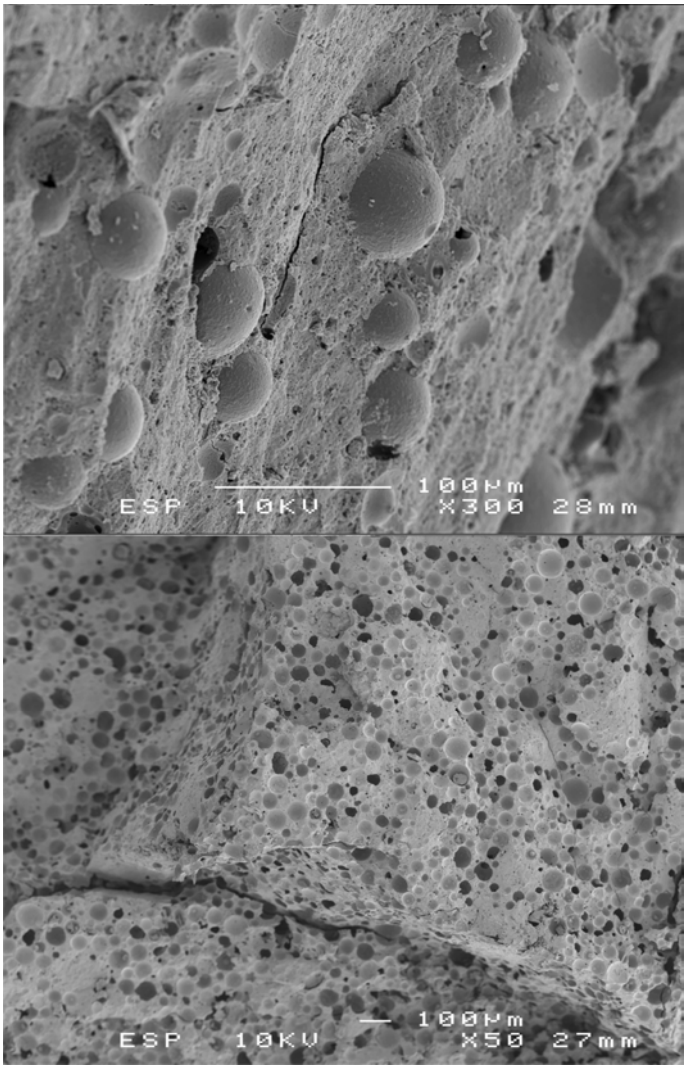



**Figure 2.** PLGA microsphere/CaP cement composites; close-up PLGA microspheres inside cement scaffold (above, original magnification 300x), 20% PLGA microsphere/CaP cement scaffold after 12 weeks *in vitro* degradation (below, original magnification 50x)

Introducing PLGA microspheres inside calcium phosphate cement was first done by Simon Jr. et al.[68]. Microspheres with a diameter of 180-360 µm were added to calcium phosphate cement

consisting of tetracalcium phosphate (TTCP), and after hardening cells were cultured on it. The resulting paste was moldable and showed good biocompatibility.

Ruhé et al.[69] and Habraken et al.[70] put PLGA microspheres with an average size of 20-40 μm inside an injectable CaP cement so that a macroporous scaffold could be formed after microsphere degradation while still having sufficient setting properties. *In vitro* experiments showed that the introduction of 20% PLGA microspheres in the CaP scaffold decreased the compression strength, injectability and increased cement setting time, but within manageable ranges[70]. Bulk erosion of the microspheres in the scaffolds was also observed, corresponding to a sudden decrease in mechanical strength. SEM-micrographs of these PLGA microsphere/CaP cement scaffolds before and after degradation are shown in Figure 2. Compared to the microporous cement, an extra pH decrease occurred due to PLGA hydrolysis that showed to increase cement resorption around the microspheres. Apparently, this pH decrease does not hamper *in vivo* applications, because subcutaneous implantation of these scaffolds showed no adverse tissue reaction during microsphere degradation[71] and also in cranial defects even bone ingrowth was observed[72, 73].

*In vitro* release characteristics with rhBMP-2[69] showed a sustained, but small release (5-10%) after 4 weeks in medium, though *in vivo* release characteristics[74] with low and high molecular weight PLGA microspheres showed higher release efficiencies (25-50%). Next to that, the low molecular weight PLGA microsphere composites showed the highest release after 28 days, indicating that the drug in this formulation exhibited less interaction with the surrounding cement. The discrepancy between *in vitro* and *in vivo* results could be due to a more dynamic flow and the occurrence of high amounts of salts and proteins like albumin *in vivo* that positively influenced the release properties.

Comparable cement composites were prepared by Schnieders et al.[75]. Scaffolds containing up to 20 wt% of microspheres were loaded with gentamycin (10-30 wt%) and showed a sustained *in vitro* release pattern, while the release pattern from separate microspheres exhibited a typical burst release. In this study mechanical properties/setting time were not affected as much due to the use of smaller microspheres.

Overall, these PLGA microsphere/calcium phosphate cement composites form a suitable macroporous scaffold with good physical/mechanical properties, a good body response and sustained release properties.

### *3.2. Protein-based polymer/ceramic composites*

Composites of CaP-ceramics and cements with protein-based polymers are investigated extensively because they can produce organic-inorganic composites with mechanical/physical and biological characteristics that are similar to human bone. Well-known examples of this group of biopolymers are collagen and gelatin.

Because collagen is one of the most abundant protein components inside the human body, it is not surprising that a lot of research has been done using collagen in a tissue-engineered construct. Next to

its high abundancy, there exist a lot of collagen types and forms that are usually retrieved by processing different animal tissues. Gelatin is a cheap and commercially available biomaterial that next to its household use gains more interest in general medicine. Gelatin is a processed form of collagen that is usually derived from pig-skin with an acidic process (type A) or from bovine origin with a basic (lime cured) process (type B). Both gelatin and collagen are degradable biopolymers which are solely degraded by proteolysis (collagenase, gelatinase). Degradation rates here are tailored by chemical crosslinking of the proteins, which makes the gelatin/collagen less accessible for proteolytic degradation. Introducing these biopolymers into a ceramic scaffold or introducing ceramics into a collagen/gelatin scaffold gives a composite with tailored degradation rates, and combines the biological- and mechanical/physical properties of both materials.

Porous gelatin/collagen scaffolds with ceramic particles are formulated by different authors[76-78], especially so-called gelatin sponges that are prepared by freeze-drying a gelatin/calcium phosphate mixture. The mechanical properties of these porous scaffolds are poor and often the gelatin/collagen is highly crosslinked for a better (biological) stability, though this can also decrease biocompatibility while crosslinking agents like glutaraldehyde can give a cytotoxic reaction[79]. Shibata et al.[78] therefore prepared a colloidal β-TCP/collagen composite that does not need any extra treatment. Du et al.[80] used collagen I as matrix for CaP mineralization to obtain a collagen/CaP composite. Here the biomimetic properties of the collagen were exploited and 60-70% CaP composites were produced with a high tensile strength. A different ceramic/gelatin porous scaffold was prepared by Tampieri et al.[81]. In this study a macroporous HA ceramic was soaked in gelatin solution, and a 10% gelatin composite was formed in which the gelatin penetrated in the bulk of the ceramic. Also crosslinker was used to slow down diffusion of gelatin out of the scaffold, however, a low concentration was applied that assured biocompatibility.

For the release of growth factors, these CaP particle/biopolymer composites have not been used too often, though the release properties of especially gelatin is well investigated and relies on an electrostatic interaction between a positively/negatively charged gelatin and a drug of the opposite charge[82]. Takahashi et al.[76] investigated gelatin sponges containing β-TCP particles that were loaded with BMP-2. These sponges were prepared by chemical crosslinking of the gelatin in the presence of different amounts of β-TCP granules; BMP-2 loading was done by simply dropping a solution of the growth-factor to freeze-dried sponges. Release was followed *in vivo* by labeling the BMP-2 with $^{125}I$, which was observed to be similar for all composites and showed a high initial release followed by a more sustained release. Bone formation was also observed with all types of composites, though high amounts of β-TCP granules made the sponge collapse during degradation leading to denser structures and less bone formation. Kim et al.[77] added gentamycin to a HA-particle containing gelatin sponge and investigated the *in vitro* drug entrapment and release as a function of crosslinking density of the gelatin, amount of HA in the sponge and drug loading. Results show that increasing the crosslinking density leads to a higher drug entrapment, a decreasing initial drug release and a more sustained

release pattern. An increase in the % of HA particles decreased the % drug entrapment, though release here is also more sustained. *In vivo* studies using a collagen scaffold with HA nanoparticles and rhBMP-2 were also performed; here a positive influence of the added drug was observed in a loaded implant model in dogs[83, 84].

Composites of gelatin/collagen with calcium phosphate cements are formulated to obtain better handling properties and to improve biological response. Bigi et al.[85-87] introduced 18% of gelatin inside calcium phosphate cement (α-TCP) and studied the biological response as well as the properties of the composite. They found an acceleration of the cement setting reaction as well as an increase in compression strength due to the high density of the gelatin. Furthermore an excellent biological response was observed with an improvement of osteoblast activity and differentiation. Fujishiro et al.[88] prepared a porous composite of a 5% and 20% gelatin containing α-TCP-based cement. Pores of 20-100 μm were prepared by mixing a gelatin gel with α-TCP powder. Results showed an increase in mechanical properties over time with the 5% composites when compared to the original cement, though with 20% gelatin the compressive strength was lower. The good biological response and increase in mechanical strength is mostly due to the biomimetic abilities of gelatin that functions as a nucleation point of CaP-crystal growth[89]. Next to gelatin, also the addition of collagen to a CaP-cement is investigated. Rammelt et al.[90] added 3% of collagen I to a nanocrystalline hydroxy-apatite cement which lead to an increased bone remodeling in rat tibia defects. Knepper-Nicolai et al.[91] added 1-2.5% collagen I to a commercial apatite cement and also observed mechanical and physical changes of the cement especially when osteocalcin was added. Hempel et al.[92] investigated the behavior of osteoblasts cultered on the same 2.5% collagen containing cement and observed that changes in the local microenvironment like the pH greatly influenced cell differentiation and proliferation on these composites.

Release properties of these collagen/cement composites were investigated by Lode et al.[93]. They studied the release of VEGF from a collagen containing apatite cement and compared it to the release pattern/VEGF activity of the normal cement. Cumulative release (25%) and burst release from the collagen cement was higher than from plain cement indicating a looser binding of the VEGF to the collagen. Additionally, the introduction of phospherine and sodium citrate to the collagen containing cement showed a positive impact on VEGF activity. This was determined by analyzing the proliferation of endothelial cells after exposing them to the diluted release medium.

Next to gelatin and collagen also other protein structures are used as composite material for CaP-ceramics and cements. Fibrin, and especially fibrin glue was applied for the introduction of ceramic particles[94, 95], or mixed together with calcium phosphate cement[96]. Studies have been performed introducing alumina and TCP-particles in a casein and soybean protein scaffold[97], and polypeptides into CaP-cement[98]. Again, main reasons for the use of these proteins were biodegradability, biomimetic properties and mechanical reinforcement.

### *3.3. Carbohydrate-based polymer/ceramic composites*

Next to protein based biopolymers, a different group of biodegradable polymers are of interest for general medicine and tissue engineering purposes, i.e. carbohydrate-based biopolymers. Most well-known are probably chitin and chitosan, a derivative of chitin, but also alginate, hyaluronan and cellulose are members of this group.

Of these polymers, chitosan is often applied together with CaP-cement or CaP-particles because it is biodegradable by lyctic enzymes and it gives a good match to natural bone with respect to mechanical properties. Regarding CaP-cement, chitosan can improve the mechanical/ physical properties as well as influence cement setting, though the compression strength of the ceramic is superior to chitosan.

Chitosan can be added to a CaP-cement as adjuvant to make the cement more injectable as was observed by Leroux et al.[99] without substantially modifying the setting reaction. Similar results were observed by Takagi et al.[100], who concluded that CPC-chitosan composites exhibited a better cohesion in water than regular tetracalcium phosphate (TTCP) cement. Xu et al.[101] observed a reinforcement of a TTCP-cement by the addition of chitosan lactate. An increase in flexural strength, work of fracture and strain-at-peak load was observed, which was explained by the difference in microstructure of the chitosan composite. Also macroporosity was added to the composite by addition of mannitol crystals[102]. This led to a dramatic decrease of the elastic modulus and flexural strength, though macroporosities up to 65% were obtained and cells show infiltration into the scaffolds.

The CPC-chitosan composites can also be applied for drug release. Takechi et al.[103] added flomoxef sodium to the liquid phase of the TTCP-cement/chitosan composite and measured release from preset disks for 3 days. Results showed a release pattern that was characterized by an initial burst, followed by a more sustained release. The total % of drug released in 24 hrs was 24-35%, where the addition of chitosan in different amounts did not influence total release after 72hrs. Even more, the release from these chitosan enriched cements did not differ significantly from the normal TTCP cement, though the maximum amount of chitosan used was 1.0%.

Similar to other polymer-ceramic composites, the opposite construction with ceramic particles in a porous chitosan composite is also formulated. Lee et al.[104] added TCP particles to chitosan sponges by freeze-drying and crosslinking a mixture of a chitosan solution and TCP. Zhang et al.[105] added HA and calcium phosphate invert glass to a chitosan sponge using a similar procedure. The added ceramic particles gave both scaffolds an extra osteogenic potential while the composite materials showed enhanced osteoblast proliferation and differentiation. Next to that, E-modulus and yield strength were reported to improve when introducing invert glass and β-TCP particles[106]. These scaffolds were also loaded with gentamycin by immersing them in gentamycin-containing PBS solutions. Release patterns showed a high burst release that was diminished by addition of the ceramic particles and a total release of > 90% after 3 weeks in release medium. A more sustained release from the particle-containing composite was observed, which was suggested to occur due to a higher extend of chitosan crosslinking. Lee et al.[107] also introduced platelet-derived growth factor (PDGF) to the chitosan-TCP

scaffolds and measured *in vitro* release by $^{125}$I-labelled PDGF and *in vivo* bone regeneration in calvarial defects in rats. The release test showed an initial burst that was followed by a slower maintained release. *In vivo*, the addition of PDGF further enhanced bone formation.

Next to chitosan, hydroxypropylmethylcellulose (HPMC, a cellulose derivative) is another carbohydrate applied in combination with ceramic particles or as an addition to calcium phosphate cement. Daculsi et al.[108] developed an injectable bone substitute comprised of BCP particles in a HPMC carrier. The materials showed to poses poor mechanical properties but a fast *in vivo* dissolution followed by replacement with newly formed bone. Crosslinking the HPMC by silanol groups (si-HPMC) renders a more stable scaffold with a comparable *in vivo* performance[109]. Burguera et al.[110] added HPMC as a gelling agent to a conventional and DCPD containing cement. Here a dramatic increase in injectability was observed after adding the HPMC, which was due to the high compressibility of the polymer.

Comparable injectable pastes comprised of amylopectin with β-TCP granules were prepared by Ongpipattanakul et al.[111]. These pastes were loaded with rhTGF-β by absorbing the growth factor on the granules and *in vitro* results showed a release of 80% after 24h incubation in serum. When implanted in a rabbit unilateral segmental defect model a positive bone response of the added TGF-β was observed after 56 days. *In vivo* release data showed a half life of the TGF-β at the implant site of 4-6 days, next to that the growth factor also remained intact for more than 21 days *in vivo* as was observed in SDS-page.

Composites of bioactive glass and starch/cellulose acetate and a mixture of methylmethacrylate/acrylic acid have been investigated by Boesel et al.[112]. Different amounts of bioactive glass were added up to 30%, which resulted in a significant improvement in E-modulus and yield strength of the material. In general no bioactivity was observed due to the inhibiting effect of the acrylic acid. Only with the highly reactive Bioglass®, calcium phosphate precipitation occurred.

Hyaluronan is another attractive carbohydrate-based polymer for tissue engineering that is present in the human body and proteolytically degradable by hyaluronidases. Bone grafts consisting of HA granules in a hyaluronan carrier were investigated by Liljensten et al.[113] who also investigated HA/phospholipid scaffolds. Comparison of these materials to HA granules without carrier material showed a significant increase in newly formed bone when implanted in circular defects in rabbit tibia.

The use of alginate in inorganic-organic scaffolds for bone tissue engineering is limited due to the poor biodegradation, though recently attempts have been made to make the alginate more biodegradable by shortening the chains and by chemical modifications[114]. Alginate has been added as a cohesion promoter for calcium phosphate cements because these materials have the tendency to disintegrate upon contact with blood/fluid. Also chitosan, as discussed before, and other hydrocarbonate biopolymers have the ability to improve cement cohesion. Khairoun et al.[115] therefore investigated the cohesion properties of a commercial HA-cement after the addition of a wide range of these polymers i.e.: hydroxyethyl starch, starch, sodium dextran sulphate, α/β/λ-cyclodextrine, alginic acid,

hyaluronic acid and chondroitine sulphate. Results for all biopolymer/cement composites show a reduced cohesion time and small changes in setting time and mechanical properties when compared to the original cement.

### *3.4. Other polymer/ceramic composites*

Next to the polymer/ceramic composites already discussed in the last three sections, composites of ceramics with different types of polymers were also formulated. Especially synthetic polyesters, other than polylactic/glycolic acid discussed previously, can be suitable carrier materials for ceramic particles. In this paragraph the characteristics of these and other polymer composites as well as their use for tissue engineering will be discussed briefly.

A composite comprised of 25% β-TCP particles in a poly(propylene fumarate) (PPF) carrier was formulated by Peter et al.[116]. PPF is a slow biodegradable and crosslinkable unsaturated linear polyester that is injectable and can provide a good mechanical support for (bone) defects. β-TCP particles were introduced for their osteoconductive properties, thereby making the PPF a more suitable material for bone tissue engineering.

A different group of ester-backbone biodegradable polymers that can be applied for bone tissue engineering purposes is the polycarbonate group. These polymers degrade hydrolytically or proteolytically by lipase, though, because it is a synthetic polymer, molecular weight and therefore degradation characteristics can be varied. Besides, there is also a wide choice of polycarbonate compounds ranging from very elastic polymers to more stiff materials. A composite of a poly(desaminotyrosyl tyrosine ethyl esther carbonate) (poly(DTE carbonate)) and CaP glass fibers ($CaO$-$P_2O_5$-$Fe_2O_3$) was formulated by Charvet et al.[117]. They installed short fibers of 2-3 mm length into a mould after which a solution of polycarbonate in methylene chloride was added. After that, the samples were heat processed and compression molded. Results showed that addition of the fibers doubles the stiffness of the composites; additional plasma coating of the fibers even improved the mechanical properties further due to a better matrix/fiber bonding.

Polyhydroxyalkanoates (PHA) form another group of biodegradable aliphatic polyesters that have their applications in a broad field of medicine. Biodegradation of these polymers can be tailored by changing the polymer length and distance between ester groups. Different polymers of these group are formulated, ranging from a stiff and brittle material (poly(3-hydroxybutyrate), poly(3HB)) to more flexible structures (poly(3-hydroxybutyrate-co-hydroxyvalerate)(poly(3HB-*co*-3HV)) and poly(3-hydroxybutyrate-*co*-hydroxyhexaoate) poly(3HB-*co*-3HHx)). Ceramic particles of β-TCP, HA and Bioglass® were added to these polymers[118], which in case of poly(3HB) and poly(3HB-*co*-3HV) led to an increase in mechanical properties and bioactivity. With the addition of hydroxyapatite particles to poly(3HB-*co*-3HHx) polymer an adverse effect was obtained, probably due to aggregations of HA particles that affected the mechanical strength and osteoblast interaction.

Poly(diol citrate)-HA microparticle composites were prepared and characterized by Qui et al.[119]. Poly(diol citrate) is an elastic polyester that is biodegradable (hydrolysis) with a degradation time ranging from a few months to a year. Composites up to 65% of HA were prepared in which an increase in HA content led to better mechanical properties (denser), a slower degradation and improved mineralization *in vitro*.

Mickiewicz et al.[120] added a range of water-soluble polymers comprising of different polyelectrolytes, poly(ethylene oxide) and bovine serum albumin (BSA) during cement setting of a brushite cement. Addition of the polycations poly(ethylenimine) and poly(allylamine hydrochloride) as well as BSA improved the mechanical properties of the cement due to a denser microstructure. For tissue engineering applications these materials can be useful due to their strengthening abilities and dissolvability, however, they should not give an adverse tissue response. The use of BSA, as already discussed in the paragraph about cements, also can be beneficial for drug release especially in low releasing calcium phosphate cements.

A composite comprised of an α-TCP based apatite cement and reinforced with polypropylene, nylon and carbon fibers was investigated by dos Santos et al.[121]. Although compressive strength decreased by addition of these fibers, an increase in toughness and tensile strength was observed. Also Xu et al.[122] added fibers consisting of either aramide, carbon, Epoxy-glass or polyglactin to a calcium phosphate cement and found an increase in tensile strength, work-of-fracture and elastic modulus. Most of these polymers, however, are not biodegradable and therefore not applicable for tissue engineering purposes.

## 4. Concluding remarks

From this review it can be concluded that there is a wide interest in the use of ceramics for bone tissue engineering purposes, whether it comprises a 100% ceramic composite, a composite of polymer with ceramic particles or addition of polymeric additives/porogens to calcium phosphate cement. The ideal scaffold parameters that were stated in the introduction therefore can be obtained using a broad range of materials and techniques. Scaffolds made of solely osteoconductive bioceramics can be feasible for tissue engineering purposes when porosity is introduced because they will allow bone ingrowth. The porosity also will increase the resorption rates, however, can decrease mechanical properties. Although tailorable within certain ranges, complete resorption of the ceramic remains difficult. Another challenge is the brittleness of the ceramic, which makes implantation for load-bearing applications complex. CaP cements/ ceramics can be applied as carriers for drugs in which release patterns and efficiencies strongly depend on the type of ceramic/cement used. In general, ceramics show a higher initial release than cements that have a more sustained release pattern. Also bioactive glass prepared by sol-gel can be used as a carrier for drugs.

The formulation of polymer/ceramic composites can compensate for the brittleness as well as improve the biodegradation and drug release of the original ceramic but compression strength and biological activity of the added polymers are often inferior.

Biopolymers like chitosan, gelatin and collagen are frequently introduced into calcium phosphate cement to improve the *in vivo* cohesion and injection properties of the cement. Occasionally these polymers are applied to mechanically reinforce the cement, which is a result of the compact microstructure that is formed after mixing both inorganic-organic materials. The degradation properties and porosity of cement can be altered by adding polymer microspheres/particles that degrade *in vivo* as was observed with PLGA-microspheres. Furthermore, PLGA microsphere and collagen containing cements have shown to be promising materials for drug release/entrapment.

Ceramic particles can also be added to a polymeric carrier to improve or introduce osteogenic properties. Scaffolds comprised of a ceramic in a polylactic, protein or carbohydrate carrier overall show higher degradation rates than pure ceramics. In addition, degradation rates can be tailored by changing the molecular weight or crosslinking density of the polymer. Compression strength of these scaffolds is less than of 100% ceramic composites; on the other hand, these polymer-based materials are not as brittle. Release patterns from drug-loaded scaffolds overall show a high initial release. The addition of higher amounts of ceramic particles to for e.g. chitosan composites or increased crosslinking of the gelatin chains decreases the initial burst release and provides a more sustained release of drugs/growth factors.

## 5. Objective of this thesis

In view of the above mentioned, the objective of this thesis was to synthesize a calcium phosphate cement that is suitable for tissue engineering purposes. Therefore, biodegradable microspheres were incorporated into the cement, which introduced macroporosity after *in situ* degradation and at the same time can be used as drug delivery vehicle for osteoinductive growth factors. The hypothesis behind this model is that during degradation of the microspheres, bone ingrowth compensates for the loss of mechanical strength and increases osteoclastic resorption of the cement. Furthermore, after introduction of the microspheres, physical and mechanical properties of the cement composites still should be sufficient for clinical application.

The research as described in this thesis deals with the development of such microsphere/calcium phosphate cement composites with respect to physical/chemical analysis, degradation and drug release. The subaims of this thesis are:

1) To formulate injectable PLGA microsphere/calcium phosphate cement composites and to investigate their *in vitro* degradation characteristics
2) To investigate the combined degradation and release mechanism of a PLGA microsphere/calcium phosphate cement composite

3) To formulate calcium phosphate cement composites with incorporated gelatin microspheres and to investigate their *in vitro* degradation characteristics
4) To formulate a porcine gelatin microsphere/calcium phosphate cement composite and to investigate its combined degradation and release mechanism.
5) To investigate the *in vitro* growth factor release from various gelatin microsphere/calcium phosphate cement composites
6) To formulate calcium phosphate cement composites with incorporated PTMC microspheres and to investigate their *in vitro* degradation characteristics
7) To evaluate the *in vivo* performance of calcium phosphate cements with incorporated PLGA, gelatin or PTMC microspheres

## 6. References


1) M.W. Barsoum, Fundamentals of Ceramics, in: R. Gibala, M. Tirrell, C.A. West (Eds.), McGraw-Hill series in Materials Science and Engineering, The McGraw-Hill Companies, inc., 1997, pp. 2

2) C.J. Whitters, R. Strang, D. Brown, R.L Clarke, R.V. Curtis, P.V. Hatton, A.J. Ireland, C.H. Lloyd, J.F. McCabe, J.W. Nicholson, S.N. Scrimgeour, J.C. Setcos, K. Sheriff, R. van Noort, D.C. Watts, D. Wood, Dental materials: 1997 literature review, J Dent. 27 (1999) 401-435

3) J.E. Block, M.R. Thorn, Clinical Indications of Calcium-Phosphate Biomaterials and Related Composites for Orthopeadic Procedures, Calcif. Tissue Int. 66 (2000) 234-238

4) S. Taksali, J.N. Grauer, A.R. Vaccaro, Material considerations for invertebral disc replacement implants, Spine J. 4 (2004) 231S-238S

5) H.S. Katti, Biomaterials in total joint replacement, Colloid Surf B Biointerfaces. 39 (2004) 133-142

6) P.F. Heini, U. Berlemann, Bone substitutes in vertebroplasty, Eur Spine J. 10 (2001) S205-S213

7) H.B. Gladstone, M.W. McDermott, D.D. Cooke, Implants for cranioplasty, Otolaryngol Clin North Am. 28(2) (1995) 381-400

8) R.Z. LeGeros, Properties of Osteoconductive Biomaterials: Calcium phosphates, Clin Orthop Rel Res. 395 (2002) 81-98

9) S.M. Kenny, M. Buggy, Bone cements and fillers: A review, J Mater Sci Mater Med. 14 (2003) 923-938

10) M.V. Thomas, D.A. Puleo, M. Al-Sabbagh, Bioactive glass three decades on., J Long Term Eff Med Implants. 15(6) (2005) 585-597

11) V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials. 26 (2005) 5474-5491

12) H. Seeherman, J.M. Wozney, Delivery of bone morphogenic proteins for orthopedic tissue regeneration, Cytokine Growth Factor Rev. 16 (2005) 329-345

13) M.P. Ginebra, T. Traykova, J.A. Planell, Calcium phosphate cements as bone drug delivery systems: A review, J Control Release. 113 (2006) 102-110

14) J.A. Jansen, J.W.M. Vehof, P.Q. Ruhé, H. Kroeze-Deutman, Y. Kuboki, H. Takita, E.L. Hedberg, A.G. Mikos, Growth factor-loaded scaffolds for bone engineering, J Control Release. 101 (2005) 127-136

15) H. Yuan, C.A. van Blitterswijk, K. de Groot, J.D. de Bruijn, Cross-species comparison of ectopic bone formation in biphasic calcium phosphate (BCP) and hydroxyapatite (HA) scaffolds, Tissue Eng. 12(6) (2006) 1607-1615

16) D.W. Hutmacher, Scaffolds in tissue engineering bone and cartilage, Biomaterials. 21 (2000) 2529-2543

17) C. Muzzarelli, R.A.A. Muzzarelli, Natural and artificial chitosan-inorganic composites, J Inorg Bioch. 92 (2002) 89-94

18) A.C. Lawson, J.T Czernuszka, Collagen-calcium phosphate composites, Proc Inst Mech Eng [H]. 212(6) (1998) 413-425

19) M. Mastrogiacomo, S. Scalione, R. Marinetti, L. Dolcini, F. Beltrame, R. Cancedda, R. Quarto, Role of scaffold internal structure on in vivo bone formation in macroporous calcium phosphate ceramics, Biomaterials. 27(17) (2006) 3230-3237

20) C.E. Wilson, J.D. de Bruijn, C.A. van Blitterswijk, A.J. Verbout, W.J. Dhert, Design and fabrication of standardized hydroxyapatite scaffolds with a defined micro-architecture by rapid prototyping for bone-tissue-engineering research, J Biomed Mater Res Part A. 68(1) (2004) 123-132

21) A. Almiral, G. Larrecq, J.A. Delgado, S. Martinez, J.A. Planell, M.P. Ginebra, Fabrication of low temperature macroporous hydroxyapatite scaffolds by foaming and hydrolysis of an alpha-CP paste, Biomaterials. 25(17) (2004) 3671-3680

22) M.C. Kruyt, C.E. Wilson, J.D. de Bruijn, C.A. van Blitterswijk, C.F. Oner, A.J. Verbout, W.J.A. Dhert, The effect of cell-based bone tissue engineering in a goat transverse process model, Biomaterials. 27 (2006) 5099-5106

23) H.P. Stallmann, C. Faber, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, In vitro gentamycin release from commercially available calcium-phosphate bone substitutes influence of carrier type on duration of the release profile, BMC Musculosketal Disorders. 7(18) (2006) 1-8

24) J. Guicheux, O. Gauthier, E. Aguado, D. Heymann, P. Pilet, S. Couillard, A. Faivre, G. Daculsi, Growth hormone-loaded macroporous calcium phosphate ceramic: *In vitro* biopharmaceutical characterization and preliminary *in vivo* study, J Biomed Mater Res Part A. 40 (1998) 560-566

25) S. Kimakhe, S. Bohic, C. Larosse, A. Reynaud, P.Pilet, B. Giumelli, D. Heymann, G. Daculsi, Biological activities of sustained polymixin B release from calcium phosphate biomaterial prepared by dynamic compaction: An *in vitro* study, J Biomed Mater Res Part A. 47 (1999) 18-27

26) J. Ziegler, U. Mayr-Wohlfahrt, S. Kessler, D. Breitig, K.-P. Günther, Adsorption and release properties of growth factors from biodegradable implants, J Biomed Mater Res Part A. 59 (2002) 422-428

27) H. Yuan, M. van den Doel, S.H. Li, K. de Groot, J.D. de Bruijn, A comparison of the osteoconductive potential of two calcium phosphate ceramics implanted intramuscularly in goats, J Mater Sci Mater Med. 13 (2002) 1272-1275

28) P. Habibovic, T.M. Sees, M.A. van den Doel, C.A. van Blitterswijk, K. de Groot, Osteoinduction by biomaterials-Physicochemical and structural influences, J Biomed Mater Res Part A. 77 (2006) 747-762

29) J.A. Jansen, E. Ooms, N. Verdonschot, J.G.C. Wolke, Injectable calcium phosphate cement for bone repair and implant fixation, Orthop Clin N Am. 36 (2005) 89-95

30) M. Bohner, U. Gbureck, J.E. Barralet, Technological issues for the development of more efficient calcium phosphate bone cements: A critical assessment, Biomaterials. 26 (2005) 6423-6429

31) L.C. Chow, S. Takagi, P.D. Costantino et al., Self-setting CaP cements, Mater Res Symp Proc. 179 (1991) 3-24

32) H.H.K. Xu, M.D. Weir, E.F. Burguera, A.M. Frasier, Injectable and macroporous calcium phosphate scaffold, Biomaterials. 27 (2006) 4279-4287

33) R.P Del Real, J.G.C. Wolke, M. Vallet-Regi, J.A. Jansen, A new method to produce macropores in calcium phosphate cements, Biomaterials. 23 (2002) 3673-3680

34) R.P Del Real, E.M. Ooms, J.G.C. Wolke, M. Vallet-Regi, J.A. Jansen, In vivo bone response to porous calcium phosphate cement, J Biomed Mater Res Part A. 65 (2003) 30-37

35) M-P. Ginebra, J-A. Delgado, I. Harr, A. Almirall, S. Del Valle, J.A. Planell, Factors affecting the structure and properties of an injectable self-setting calcium phosphate foam, J Biomed Mater Res Part A. 80(2) (2006) 351-361

36) D.P Link, J. van den Dolder, J.G.C. Wolke, J.A. Jansen, The cytocompatibility and early osteogenic characteristics of an injectable calcium phosphate cement, Tissue Engineering. 13(3) (2007)

37) E.J Blom, J. Klein-Nulend, J.G.C. Wolke, K. Kurashina, M.A.J. van Waas, E.H. Burger, Transforming growth factor-β1 incorporation in an α-tricalcium phosphate monoxide cement: release characteristics and physicochemical properties, Biomaterials. 23 (2002) 1261-1268

38) P.Q. Ruhé, O.C. Boerman, F.G.M. Russel, A.G. Mikos, P.H.M. Spauwen, J.A. Jansen, *In vivo* Release of rhBMP-2 Loaded Porous Calcium Phosphate Cement Pretreated with Albumin, J Mater Sci Mater Med. 17(10) (2006) 919-927

39) H.P. Stallmann, C. Faber, E.T. Slotema, D.M. Lyaruu, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11(hLF1-11) from calcium phosphate bone substitutes, J Antimicrob Chemother. 52 (2003) 853-855

40) H. Seeherman, R. Li, M. Bouxsein, H. Kim, X.J. Li, E.A. Smith-Adaline, M. Aiolova, J.M. Wozney, rhBMP-2/calcium phosphate matrix accelerates osteotomy-site healing in a nonhuman primate model at multiple treatment times and concentrations, J Bone Joint Surg Am. 88(1) (2006) 144-160

41) P.Q. Ruhé, H.C. Kroeze-Deutman, J.G.C. Wolke, P.H.M. Spauwen, J.A. Jansen, Bone inductive properties of rhBMP-2 loaded porous calcium phosphate cement implants in cranial defects in rabbits, Biomaterials. 25 (2004) 2123-2132

42) H.C. Kroese-Deutman, P.Q. Ruhé, P.H.M. Spauwen, J.A. Jansen, Bone inductive properties of rhBMP-2 loaded porous calcium phosphate cement implants inserted at an ectopic site in rabbits, Biomaterials. 26 (2005) 1131-1138

43) E.J. Blom, J. Klein-Nulend, L. Yin, M.A. van Waas, E.H. Burger, Transforming growth factor-beta1 incorporated in calcium phosphate cement stimulates osteotransductivity in rat calvarial bone defects, Clin Oral Implants Res. 12(6) (2001) 609-616

44) L.L. Hench, O. Anderson, In: An introduction to Bioceramics, Chapter 3: Bioactive glasses, pp 41-61

45) J. Zhong, D.C. Greenspan, Processing and Properties of Sol-Gel Bioactive Glasses, J Biomed Mater Res Part B: Appl Biomater. 53 (2000) 694-701

46) T. Kokubo, H-M Kim, M. Kawashita, Novel bioactive materials with different mechanical properties, Biomaterials. 24 (2003) 2161-2175

47) Y. Ebisawa, F. Miyaji, T. Kokubo, K. Ohura, T. Nakamura, Bioactivity of ferrimagnetic glass-ceramics in the system $FeO$-$Fe_2O_3$-$CaO$-$SiO_2$, Biomaterials. 18 (1997) 1277-1284

48) H. Yuan, J.D. de Bruijn, X. Zhang, C.A. van Blitterswijk, K. de Groot, Bone Induction by Porous Glass Ceramics Made from Bioglass®, J Biomed Mater Res Part B: Appl Biomater. 58 (2001) 270-276

49) Q,Z. Chen, I.D. Thompson, A.R. Boccoccini, 45S5 Bioglass®-derived glass-ceramic scaffolds for bone tissue engineering, Biomaterials. 27 (2006) 2414-2425

50) E.A.B.E. Kaufmann, P. Ducheyne, I.M. Shapiro, Effect of varying physical properties of porous, surface modified bioactive glass 45S5 on osteoblast proliferation and maturation, J Biomed Mater Res Part A. 52 (2000) 783-796

51) P. Sepulveda, J.R. Jones, L.L. Hench, *In vitro* dissolution of melt-derived 45S5 and sol-gel derived 58S bioactive glasses, J Biomed Mater Res Part A. 61 (2002) 301-311

52) M.G. Cerruti, D. Greenspan, K. Powers, An analytical model for the dissolution of different particle size samples of Bioglass® in TRIS-buffered solution, Biomaterials. 26 (2005) 4903-4911

53) E.M. Santos, S. Radin, P. Ducheyne, Sol-gel derived carrier for the controlled release of proteins, Biomaterials. 20 (1999) 1695-1700

54) Z.R. Domingues, M.E. Cortés, T.A. Gomes, H.F. Diniz, C.S. Freitas, J.B. Gomes, A.M.C. Faria, R.D. Sinisterra, Bioactive glass as a drug delivery system of tetracycline and tetracycline associated with β-cyclodextrin, Biomaterials. 25 (2004) 327-333

55) M. Navarro, M.P. Ginebra, J.A. Planell, S. Zeppetelli, L. Ambrosio, Development and cell response of a new biodegradable composite scaffold for guided bone regeneration, J Mater Sci Mater Med. 15 (2004) 419-422

56) T. Niemela, H. Niiranen, K. Kellomaki, P. Tormala, Self-reinforced composites of bioresorbable polymer and bioactive glass with different bioactive glass contents. Part I: initial mechanical properties and bioactivity, Acta Biomater. 1(2) (2005) 235-242

57) J. Yao, S. Radin, G. Reilly, P.S. Leboy, P. Ducheyne, Solution-mediated effect of bioactive glass in poly(lactic co glycolic)-bioactive glass composites on osteogenesis of marrow stromal cells, J Biomed Mater Res Part A. 75(4) (2005) 794-801

58) R. Zhang, P.X. Ma, Poly(α-hydroxyl acids)/hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology, J Biomed Mater Res Part A. 44 (1999) 446-455

59) Y. Shikinami, M. Okuno, Bioresorbable devices made of forged composites of hydroxyapatite (HA) particles and poly-L-lactide (PLLA): Part I. Basic characteristics, Biomaterials. 20 (1999) 859-877

60) Y. Shikinami, Y. Matsusue, T. Nakamura, The complete process of bioresorption and bone replacement using devices made of forged composites of raw hydroxyapatite particles/poly l-lactide (F-u-HA/PLLA), Biomaterials. 26 (2005) 5542-5551

61) M.J. Mondrinos, R. Dembzynski, L. Lu, V.K.C. Byrapogu, D.M. Wootton, P.I. Lelkes, J. Zhou, Porogen-based solid freeform fabrication of polycaprolactone-calcium phosphate scaffolds for tissue engineering, Biomaterials. 27 (2006) 4399-4408

62) N. Ignjatovic, P. Ninkov, V. Kojic, M. Bokurov, V. Srdic, D. Krnojelac, S. Selakovic, D. Uskokovic, Cytotoxicity and fibroblast properties during in vitro test of biphasic calcium phosphate/poly-dl-lactide-co-glycolide biocomposites and different phosphate materials, Microsc Res Tech. 69 (2006)

63) A.M.A. Ambrosio, J.S. Sahota, Y. Khan, C.T. Laurencin, A novel amorphous calcium phosphate polymer ceramic for bone repair: I. synthesis and characterization, J Biomed Mater Res Part B: Appl Biomater. 58 (2001) 295-301

64) A.A. Ignatius, O. Betz, P. Augat, L.E. Claes, In vivo investigation on composites made of resorbable ceramics and poly(lactide) used as bone graft substitutes, J Biomed Mater Res Part A. 58(6) (2001) 701-709

65) I. Soriano, C. Evora, Formulation of calcium phosphates /poly (*d,l*-lactide) blends containing gentamycin for bone implantation, J Control Release. 68 (2000) 121-134

66) L. Lu, G.N. Stamatas, A.G. Mikos, Controlled release of transforming growth factor beta1 from biodegradable polymer microparticles, J Biomed Mater Res Part A. 50 (2000) 440-451

67) G. Schwach, N. Oudry, S. Delhomme, M. Luck, H. Lindner, R. Gurny, Biodegradable microparticles for sustained release of a new GnRH antagonist—part I: Screening commercial PLGA and formularion technologies, Eur J Pharm Biopharm. 56(3) (2003) 327-336

68) C.G. Simon Jr., C.A. Khatri, S.A. Wight, F.W. Wang, Preliminary report on the biocompatibility of a moldable, resorbable, composite bone graft consisting of calcium phosphate cement and poly(lactide-co-glycolide) microspheres, J Orthop Res. 20 (2002) 473-482

69) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, rhBMP-2 release from injectable poly(DL-lactic-*co*-glycolic acid)/calcium phosphate cement composites, J Bone Joint Surg Am. 85A *Suppl* 3 (2003) 75-81

70) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics, J Biomat Sci Polym Edn. 17(9) (2006) 1057-1074

71) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, Biocompatibility and degradation of poly(DL-lactic-*co*-glycolic acid)/Calcium phosphate cement composites, J Biomed Mater Res Part A. 74 (2005) 533-544

72) D.P. Link, J. van den Dolder, W.J.F.M. Jurgens, J.G.C. Wolke, J.A. Jansen, Mechanical evaluation of implanted calcium phosphate cement incorporated with PLGA microparticles, Biomaterials. 27 (2006) 4941-4947

73) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, Porous poly(DL-lactic-*co*-glycolic acid)/calcium phosphate cement composite for reconstruction of bone defects, Tissue Engineering. 12(4) (2006) 789-800

74) P.Q. Ruhé, O.C. Boerman, F.G.M. Russel, P.H.M. Spauwen, A.G. Mikos, J.A. Jansen, Controlled release of rhBMP-2 loaded poly(DL-lactic-*co*-glycolic acid)/Calcium phosphate cement composites in vivo, J Control Release. 106(1-2) (2005) 162-171

75) J. Schnieders, U. Gbureck, R. Thull, T. Kissel, Controlled release of gentamycin from calcium phosphate-poly(lactic acid-*co*-glycolic acid) composite bone cement, Biomaterials. 27 (2006) 4239-4249

76) Y. Takahashi, M. Yamamoto, Y. Tabata, Enhanced osteoinduction by controlled release of bone morphogenic protein-2 from biodegradable sponge composed of gelatin and β-tricalcium phosphate, Biomaterials. 26 (2005) 4856-4865

77) H-W. Kim, J.C. Knowles, H-E. Kim, Hydroxyapatite and gelatin composite foams processed via novel freeze-drying and crosslinking for use as temporary hard tissue scaffolds, J Biomed Mater Res Part A. 72 (2004) 136-145

78) Y. Shibata, H. Yamamoto, T. Miyazaki, Colloidal β-tricalcium phosphate prepared by discharge in a modified body fluid facilitates synthesis of collagen composites, J Dent Res. 84(9) (2005) 827-831

79) F.H. Lin, C.H. Yao, J.S. Sun, H.C. Liu, C.W. Huang, Biological effects and cytotoxicity of the composite composed by tricalcium phosphate and glutaraldehyde-cross-linked gelatin, Biomaterials. 19 (1998) 905-917

80) C. Du, F.Z. Cui, W. Zhang, Q.L. Feng, X.D. Zhu, K. de Groot, Formation of calcium phosphate/collagen composites through mineralization of collagen matrix, J Biomed Mater Res Part A. 50(4) (2000) 518-527

81) A. Tampieri, G. Celotti, E.Landi, M. Montevecchi, N. Rovieri, A. Bigi, S. Panzavolta, M.C. Sidoti, Porous phosphate-gelatin composite as bone graft with drug delivery function, J Mater Sci Mater Med. 14 (2003) 623-627

82) M. Yamamoto, Y. Ikada, Y. Tabata, Controlled release of growth factors based on biodegradation of gelatin hydrogel, J Biomater Sci Polym Edn. 12(1) (2001) 77-88

83) S. Itoh, M. Kikuchi, Y. Koyama, K. Takakuda, K. Shinomiya, J. Tanaka, Development of a hydroxyapatite/collagen nanocomposite as a medical device, Cell Transplant. 13(4) (2004) 451-461

84) S. Itoh, M. Kikuchi, K. Takakuda, Y. Koyama, H.N. Matsumoto, S. Ichinose, J. Tanaka, T. Kawauchi, K. Shinomiya, J Biomed Mater Res Part A. 54(3) (2001) 445-453

85) A. Bigi, B. Bracci, S. Panzavolta, Effect of added gelatin on the properties of calcium phosphate cement, Biomaterials. 25 (2004) 2893-2899

86) A. Bigi, P. Torricelli, M. Fini, B. Bracci, S. Panzavolta, L. Sturba, R. Giardino, A biomimetic gelatin-calcium phosphate bone cement, Int J Artif Organs. 27(8) (2004) 664-673

87) A. Bigi, S. Panzavolta, L. Sturba, P. Torricelli, M. Fini, R. Giardino, Normal and osteopenic bone-derived osteoblast response to a biomimetic gelatin-calcium phosphate bone cement, J Biomed Mater Res Part A. 78 (2006) 739-745

88) Y. Fujishiro, K. Takahashi, T. Sato, Preparation and compressive strength of α-tricalcium phosphate/gelatin gel composite cement, J Biomed Mater Res Part A. 54 (2001) 525-530

89) A. Bigi, E. Boanini, S. Panzavolta, H. Roveri, Biomimetic growth of hydroxyapatite on gelatin films doped with sodium polyacrylate, Biomacromolecules. 1(4) (2000) 752-756

90) S. Rammelt, E. Schulze, M. Witt, E. Petsch, A. Biewener, W. Pompe, H. Zwipp, Collagen type I increases bone remodeling around hydroxyapatite implants in the rat tibia, Cells Tissues Organs. 178(3) (2004) 146-157

91) B. Knepper-Nicolai, A. Reinstorf, I. Holfinger, K. Flade, R. Wenz, W. Pompe, Influence of osteocalcin and collagen I on the mechanical and biological properties of Biocement D, Biomol Eng. 19 (2002) 227-231

92) U. Hempel, A. Reinstorf, M. Pompe, U. Fisher, M. Gelinski, W. Pompe, K.W. Wenzel, Proliferation and differentiation of osteoblasts on Biocement D modified with collagen type I and citric acid, J Biomed Mater Res Part B: Appl Biomater. 71B (2004) 130-143

93) A. Lode, C. Wolf-Brandstetter, A. Reinstorf, A. Bernhardt, U. König, W. Pompe, M. Gelinski, Calcium phosphate bone cements, functionalized with VEGF: Release kinetics and biological activity, J Biomed Mater Res Part A. (2007)

94) D. Le Nihouannen, L.L. Guehennec, T. Rouillon, P. Pilet, M. Bilban, P. Layrolle, G. Daculsi, Micro-architecture of calcium phosphate granules and fibrin glue composites for bone tissue engineering, Biomaterials. 27 (2006) 2716-2722

95) F. Jegoux, E. Goyenvalle, M. Bagot D'arc, E. Aguado, G. Daculsi, In vivo biological performance of composites combining micro-macroporous biphasic calcium phosphate granules and fibrin sealant, Arch Orthop Trauma Surg. 125(3) (2005) 153-159

96) U. Knesser, A. Voogd, J. Ohnolz, O. Buettner, L. Stangenberg, Y.H. Zhang, G.B. Stark, D.J. Schaefer, Fibrin gel-immobilized primary osteoblasts in calcium phosphate bone cement: in vivo evaluation with regard to application as injectable biological bone substitute, Cells Tissues Organs. 179(4) (2005) 158-169

97) C.M. Vaz, M. Fossen, R.F. van Tuil, L.A. de Graaf, R.L. Reis, A.M. Cunha, Casein and soybean protein-based thermoplastics and composites as alternative biodegradable polymers for biomedical applications, J Biomed Mater Res Part A. 65 (2003) 60-70

98) J. Lin, S. Zhang, T. Chen, C. Liu, S. Lin, X. Tian, Calcium phosphate cement reinforced by polypeptide copolymers, J Biomed Mater Res B: Appl Biomater. 76 (2006) 432-439

99) L. Leroux, Z. Hatim, M. Freche, J.L. Lacout, Effects of various adjuvants (lactic acid, glycerol and chitosan) on the injectability of a calcium phosphate cement, Bone. 25(2 Suppl) (1999) 31S-34S

100) S. Takagi, L.C. Chow, S. Hirayama, F.C. Eichmiller, Properties of elastomeric calcium phosphate cement-chitosan, Dent Mater. 19(8) (2003) 797-804

101) H.H.K Xu, J.B. Quinn, S. Takagi, L.C. Chow, Processing and properties of strong and non-rigid calcium phosphate cement, J Dent Res. 81(3) (2002) 219-224

102) H.H.K Xu, C.G. Simon Jr., Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility, Biomaterials. 26 (2005) 1337-1348

103) M. Takechi, Y. Miyamoto, Y. Momota, T. Yuasa, S. Tatehara, M. Nagayama, K. Ishikawa, K. Suzuki, The *in vitro* antibiotic release from anti-washout apatite cement using chitosan, J Mater Sci Mater Med. 13 (2002) 973-978

104) Y.M. Lee, Y.J. Park, S.J. Lee, Y. Ku, S.B. Han, S.M. Choi, P.R. Klokkevold, C.P Chung, Tissue engineered bone formation using chitosan/tricalcium phosphate sponges, J Periodontol. 71(3) (2000) 410-417

105) Y. Zhang, M. Zhang, Cell growth and function on calcium phosphate reinforced chitosan scaffolds, J Mater Sci Mater Med. 15 (2004) 255-260

106) Y. Zhang, M. Zhang, Calcium phosphate/chitosan composite scaffolds for controlled *in vitro* antibiotic drug release, J Biomed Mater Res Part A. 62 (2002) 378-386

107) Y.M. Lee, Y.J. Park, S.J. Lee, Y. Ku, S.B. Han, P.R. Klokkevold, C.P Chung, The bone regenerative effect of platelet-derived growth factor-BB delivered with a chitosan/tricalcium phosphate sponge carrier, J Periodontol. 71(3) (2000) 418-424

108) G. Daculsi, P. Weiss, J.-M. Bouler, O. Gauthier, F. Millot, E. Aguado, Biphasic calcium phosphate/hydrosoluble polymer composites: a new concept for bone and dental substitution biomaterials, Bone. 25(2) (1999) 59S-61S

109) B.H. Fellah, P. Weiss, O. Gauthier, T. Rouillon, P. Pilet, G. Daculsi, P. Layrolle, Bone repair using a new injectable self-crosslinkable bone substitute, J Orthop Res. 24(4) (2006) 628-635

110) E.F. Burguera, H.H. Xu, M.D. Weir, Injectable and rapid-setting calcium phosphate bone cement with dicalcium phosphate dihydrate, J Biomed Mater Res Part B: Appl Biomater. 77(1) (2006) 126-134

111) B. Ongpipattanakul, T. Nyugen, T.F. Zioncheck, R. Wong, G. Osaka, L. DeGuzman, W.P. Lee, L.S. Beck, Development of tricalcium phosphate/amylopectin paste combined with recombinant human transforming growth factor beta 1 as a bone defect filler, J. Biomed Mater Res Part A. 36 (1997) 295-305

112) J.F. Boesel, M.H.V. Fernandez, R.L. Reis, The behavior of novel hydrophilic composite bone cements in simulated body fluids, J Biomed Mater Res Part B: Appl Biomater. 70 (2004) 368-377

113) E.L Liljensten, A.G. Attaelmanan, C. Larsson, H. Ljusberg-Wahren, N. Danielsen, J.M. Hirsch, P. Thomsen, Hydroxyapatite granule/carrier composites promote new bone formation in cortical defects, Clin Implant Dent Relat Res. 2(1) (2000) 50-59

114) A.D. Augst, H.J. Kong, D.J. Mooney, Alginate hydrogels as biomaterials, Macromol Biosci. 6(8) (2006) 623-633

115) I. Khairoun, F.C.M. Driessens, M.G. Boltong, J.A. Planell, R. Wenz, Addition of cohesion promotors to calcium phosphate cements, Biomaterials. 20 (1999) 393-398

116) S.J. Peter, P. Kim, A.W. Yasko, M.J. Yaszemski, A.G. Mikos, crosslinking characteristics of an injectable poly(propylene fumarate)/β-tricalcium phosphate paste and mechanical properties of the crosslinked composite for use as a biodegradable bone cement, J Biomed Mater Res Part A. 44 (1999) 314-321

117) J.L Charvet, J.A. Cordes, H. Alexander, Mechanical and fracture behaviour of a fiber-reinforced bioabsorbable material for orthopeadic applications, J Mater Sci Mater Med. 11 (2000) 101-109

118) S.K. Misra, S.P Valappil, I. Roy, A.R. Boccaccini, Polyhydroxyalkanoate (PHA)/inorganic phase composites for tissue engineering applications, Biomacromolecules. 7(8) (2006) 2249-2258

119) H. Qiu, J. Yiang, P. Kodali, J. Koh, G.A. Ameer, A citric acid based hydroxyapatite composite for orthopeadic implants, Biomaterials. 27 (2006) 5845-5854

120) R.A. Mickiewicz, A.M. Mayes, D. Knaack, Polymer-calcium phosphate cement composites for bone substitutes, J Biomed Mater Res. 61 (2002) 581-592

121) L.A. dos Santos, R.G. Carrodéguas, A.O. Boschi, A.C.F. de Arruda, Fiber-enriched double-setting calcium phosphate bone cement, J Biomed Mater Res Part A. 65 (2003) 244-250

122) H.H. Xu, F.C. Eichmiller, A.A. Guiseppetti, Reinforcement of a self-setting calcium phosphate cement with different fibers, J Biomed Mater Res Part A. 52(1) (2000) 107-114

# CHAPTER 2

## INJECTABLE PLGA MICROSPHERE / CALCIUM PHOSPHATE CEMENTS: PHYSICAL PROPERTIES AND DEGRADATION CHARACTERISTICS

*W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen*

J Biomater Sci Polym Ed. 17 (2006) 1057-1074

## 1. Introduction

Calcium phosphate (CaP) compounds are widely used as a bone substitute in the field of dentistry, orthopedic and reconstructive surgery. Especially the hydroxyapatite (HA) based compounds have an excellent biocompatibility and show some osteoconductive characteristics[1]. They are usually applied in the form of blocks or granules. Unfortunately, there are difficulties shaping these CaP blocks during surgery while they have to fit in the bone defect. In granulate form, shaping of CaP ceramics is easier, but a disadvantage is the migration of the ceramic particles. Calcium phosphate cements seem to overcome these problems[2]. These materials are composed of a powder component that is mixed with a fluid (hardener). They are injectable and can be molded *in situ*. Further, they set at body temperature and can even be shaped before complete setting. Besides the clinical advantage, CaP cements can be extremely biocompatible. Despite this, the resorption rate of these CaP cements is too low and the cement might take years to fully disappear.

To increase degradation rates, macroporosity can be introduced to the cement. In literature some methods are described to introduce macroporosity to a CaP cement like the introduction of water-soluble mannitol crystals[3-4], and a $CO_2$-gas bubble method[5-6]. *In vivo* experiments with pre-fabricated scaffolds using this last method showed high resorption rates, however, the cement paste obtained was not injectable. Another method to introduce macroporosity into a calcium phosphate cement is the application of biodegradable polymer microspheres[7-8]. When these microspheres are eroded a porous scaffold is obtained. Many types of polymer can be used for the production of the microspheres[9-14]. PLGA (poly(D,L-lactic-co-glycolic) acid) has gained most interest for microsphere use as it can provide sustained drug delivery and has a long clinical history. PLGA degrades by means of hydrolysis. Here the diffusion of the aqeous phase in the PLGA is faster than the degradation of the polymer chains and mass loss occurs instantaniously through the PLGA, which is also called bulk erosion[15]. An *in vitro* study with PLGA implants showed 90% polymer degradation within four to six weeks and complete erosion within eight weeks, depending on the porosity of the PLGA implant[16]. PLGA degradation is influenced by various factors like the polymer chain length, lactic to glycolic (L/G) ratio and characteristics of the surrounding medium[17].

The goal of this initial study was to investigate some PLGA microsphere/CaP formulations for their injectability and setting/cohesion properties. Also a degradation study was done in which the polymer and cement degradation/erosion was followed by means of a pH, mass and molecular weight assay and visualized by scanning electron microscopy. Mechanical properties of the PLGA/CaP samples like the compression strength and E-modulus were determined.

## 2. Materials and Methods

### *2.1. Materials*

$P_{DL}LGA$ (Purasorb®, Purac, Gorinchem, Netherlands) prepared by a stannous octoate ($Sn(Oct)_2$) initiating system with a molecular weight ($M_w$) of 48.0 ± 1.6 kg/mol and a lactic to glycolic acid ratio

of 50:50 was used for the microsphere synthesis. The polydispersity index (PI) of the polymer was 2.34 ± 0.07. Poly vinylic alcohol (PVA, 88% hydrolyzed, MW 22000, Acros Organics, Geel, Belgium) was used as stabilizer during the microsphere preparation. The calcium phosphate cement (Calcibon®, Biomet Merck, Darmstadt, Germany) consisted of 61% α-TCP, 26% $CaHPO_4$, 10% $CaCO_3$ and 3% precipitated HA. The cement liquid applied was a 1% aqueous solution of $Na_2HPO_4$.

### *2.2. Methods*

#### *2.2.1. Preparation PLGA microspheres*

PLGA microspheres were prepared using a water-in-oil-in-water (w/o/w)-double emulsion solvent evaporation technique. 1.0 g of PLGA was solved in 4 ml of dichloromethane (DCM) inside a 50 ml PP tube. 500µl of demineralized water ($ddH_2O$) was added while vortexing vigorously for 1 min, subsequently 6 ml of a 0.3% PVA solution was added and vortexing was continued for another 1 min. The content of the 50 ml tube was transferred to a stirred 1000 ml beaker and another 394 ml of 0.3% PVA was added slowly. This was directly followed by adding 400 ml of a 2% isopropylic alcohol (IPA) solution. The suspension was stirred for 1 hr. The spheres were allowed to settle for 15 min and the solution was decanted. The suspension left was centrifuged, and the clear solution at the top was decanted. 5 ml of $ddH_2O$ was added, the spheres were washed, centrifuged and the solution was aspirated. Finally the spheres were frozen, freeze-dryed for 24 hr and stored under argon at -20°C.

#### *2.2.2. Preparation of PLGA/CaP samples*

Samples were made by adding the PLGA microspheres to the CaP cement powder inside a 2 ml plastic syringe, subsequently adding the 1% $Na_2HPO_4$ to the mixture and stirring the contents vigorously for 30 sec (Silamat® mixing apparatus, Vivadent, Schaan, Liechtenstein). Subsequently the mixture was injected inside a teflon mould (cylinders 4.5 x 9 mm). The amounts of the constituents for the different formulations, as well as for the microporous cement, are given in Table 1. A liquid/powder (L/P) ratio of 0.32 was applied, except for the 20/80 composites were a higher amount of liquid was used. From each syringe 4-5 samples were obtained. These samples were dried in a furnace at 40°C for 1 hr, and stored at room temperature.

**Table 1.** Ingredients for the different PLGA/CaP formulations

| | CaP (mg) | PLGA mp's (mg) | 1% $Na_2HPO_4$ (µl) |
|---|---|---|---|
| Microporous cement | 1000 | 0 | 320 |
| 10/90 PLGA/CaP | 900 | 100 | 290 |
| 20/80 PLGA/CaP | 800 | 200 | 300 |

*2.2.3. Morphology analysis*

The morphology of the samples was determined by observation on a scanning electron microscope (SEM) (JEOL 6400-LINK AN 10000 at 10 kV). The samples were mounted on aluminium stubs using Carbon tape and sputter-coated with gold-palladium prior to examination.

*2.2.4. Size distribution*

The size distribution of the PLGA microspheres was determined via image analysis. Spheres were suspended in $H_2O$ and pictures were taken with an optical microscope (Leica/Leitz DM RBE Microscope system, Leica Microsystems AG, Wetzlar, GER), after which the size distribution was determined using digital image software (Leica Qwin®, Leica Microsystems AG, Wetzlar, GER).

*2.2.5. Injectability*

Injectability tests were done on the different PLGA/CaP samples according to Khairoun et al[18]. Briefly, after mixing the microspheres with the cement/liquid, the syringe, with an orifice diameter of 1.7 mm, was fitted vertically in a fixture and put under the platen of a tensile bench set in tension mode (Fig.1). 25 seconds after mixing the cement, compressive force was applied to the syringe and recorded as a function of the plunger travel length. All tests were performed in threefold.

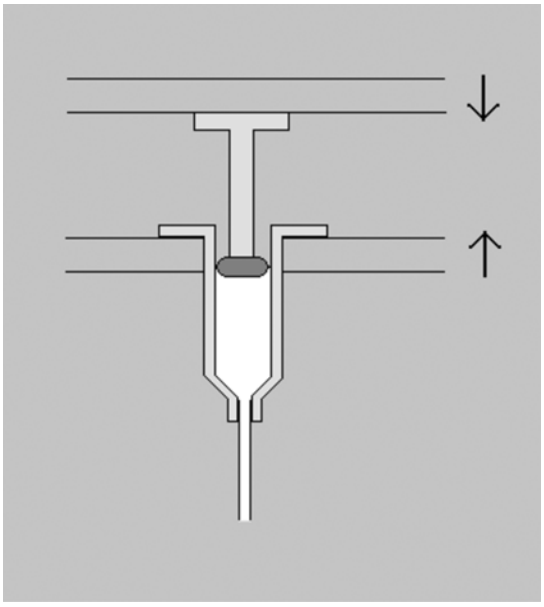


**Figure 1.** Injectability test

*2.2.6. Setting time*

The initial and final setting time of the various cement formulations was assessed using custom available Gillmore needles (ASTM C266[19]). For this a bronze block was used as mould containing 6 holes (6 mm in diameter, 12 mm in height). The mould was placed in a water bath at body temperature

(37˚C). Samples of each formulation were mixed and injected into the mould in a retrograde fashion, after which the initial and final setting time was determined. Tests were done in threefold.
For the determination of the cohesive properties of the different formulations, samples were injected into Ringer's solution at 37°C. During the cement setting time it was inspected whether the cement/composite retained its original configuration. Any anomalies were recorded.

*2.2.7. Porosity*

Of the different cement formulations the (macro)porosity was determined. In this experiment macroporosity can be regarded as the porosity of the cement formulations in which the pores are caused by (the erosion of) the PLGA microspheres. The PLGA/CaP samples therefore were weighed before they were placed in a furnace at 650°C for 2 h. After that they were weighed again, in which the difference in weight is the polymer mass content of the samples. The test was performed in threefold. Equation 1 and 2 were derived for the calculation of the total porosity and the macroporosity.

$$\varepsilon_{tot} = (1 - \frac{m_{burnt}}{V * \rho_{HAP}}) * 100\% \qquad \textit{Equation 1}$$

$$\varepsilon_{macro} = (1 - \frac{m_{burnt}}{m_{microporous}}) * 100\% \qquad \textit{Equation 2}$$

*Legend*

| | | |
|---|---|---|
| $\varepsilon_{tot}$ | = | total porosity (%) |
| $\varepsilon_{macro}$ | = | macroporosity (%) |
| $m_{burnt}$ | = | average mass sample (after burning out polymer) (g, n=3) |
| $m_{microporous}$ | = | average mass microporous sample (g, n=3) |
| V | = | volume sample ($cm^3$) |
| $\rho_{HAP}$ | = | density hydroxy apatite ($g/cm^3$) |

*2.2.8. Degradation study*

For the degradation study, PLGA/CaP and microporous cement samples were prepared as described earlier. The samples were placed in 3 ml of phosphate-buffered saline (PBS, pH 7.4) and incubated at 37˚C in a water bath on a shaker table (70 rpm) for twelve weeks. At week 2, 4, 6, 9 and 12, 5 specimens of each cement formulation were subjected to analysis. For the compression test and E-modulus 3 specimens were tested and samples after 3 days in Ringer's solution were taken as a reference.

<u>pH measurements</u>

Directly after removal of the samples from the waterbath, the pH of the PBS medium was measured.

<u>Mass loss quantification</u>

The samples were vacuum dried overnight before measuring the mass. The mass loss of the samples was calculated using Equation 3. The mass loss of the PLGA microspheres inside the samples was also

determined using Equation 4, which is a derivation of Equation 3 under the assumption of an equal cement degradation in both microporous/composite samples.

$$R_L = \frac{M_0 - M_n}{M_0} * 100\% \qquad \textit{Equation 3}$$

$$R_{L,Pol} = \frac{L_{comp,n} - b \cdot M_{comp,0} \dfrac{L_{micro,n}}{M_{micro,0}}}{a \cdot M_{comp,0}} * 100\% \qquad \textit{Equation 4}$$

*Legend*

| | | |
|---|---|---|
| $R_L$ | = | Mass loss sample on t = n (%) |
| $R_{L,Pol}$ | = | Mass loss polymer on t = n (%) |
| $M_0$ | = | Mass sample on t = 0 (g) |
| $M_n$ | = | Mass sample on t = n (g) |
| $M_{comp,0}$ | = | Mass composite sample on t = 0 (g) |
| $M_{micro,0}$ | = | Mass microporous cement on t = 0 (g) |
| $L_{comp,n}$ | = | Mass loss composite sample on t = n (g) |
| $L_{micro,n}$ | = | Mass loss microporous cement on t = n (g) |
| a | = | Mass fraction polymer on t=0 |
| b | = | Mass fraction microporous cement on t=0 |

Molecular weight analysis

The molecular weight of the PLGA polymer inside the PLGA/CaP samples was monitored using gel permeation chromatography (GPC). The system comprises of a Hitachi L2130 HPLC pump, a Hitachi L-2400 UV detector and a Merck/Hitachi L-2490 RI Detector (Hitachi corp., Tokyo, JPN). The GPC column used was a 4.6*300 mm Waters Styragel® HR 4E column with a Waters 4.6*30 mm Styragel® guard colum (Waters Corp., Milford, MA, USA). Tetrahydrofuran (THF) was used as eluent at a flow rate of 0.35 ml/min. Polystyrene (PS) standards (SM-105, Shodex corp., Tokyo, JPN) were used to obtain a primairy calibration curve. For the calculation of the $M_w$ of the PLGA, Mark-Houwink constants of PLGA 50:50 (a = 0.761, K = $1.07*10^{-2}$, THF, 22.5°C)[20] and PS (a = 0.717, K = $1.17*10^{-2}$, THF, 25°C) were used. To extract the PLGA, 100 mg of the samples was grinded and put in a sample flask together with 3 ml of THF. The suspension was put on a shaker table for 2 h and was purified using Waters 13 mm GHP 0.2µm Acrodisk® filters (Waters Corp., Milford, MA, USA).

Mechanical characteristics

Samples were placed in a testing bench (858 MiniBionixII®, MTS Corp., Eden Prairie, MN, USA) and compressive strength and E-modulus in the longitudinal direction (parallel to the long axis) of the specimens was measured at 0.5 mm/min crosshead speed.

Morphology

The morphology of the different cement formulations was determined using SEM. For this purpose 3 intersections were used that were parallel to the long axis of the samples. These samples were obtained from the compression test. Degradation behaviour of the PLGA microspheres inside the samples was visualized at higher magnifications.

*2.2.9. Statistical analysis*

Data were arranged as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat® (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

### *3.1. Characterisation PLGA microspheres*

Figure 2 shows a SEM-micrograph and size distribution of microspheres synthesized using the w/o/w double emulsion method. The spheres have a spherical and smooth appearance, and do not agglomerate. The average size of the microspheres is 33μm ± 17μm.

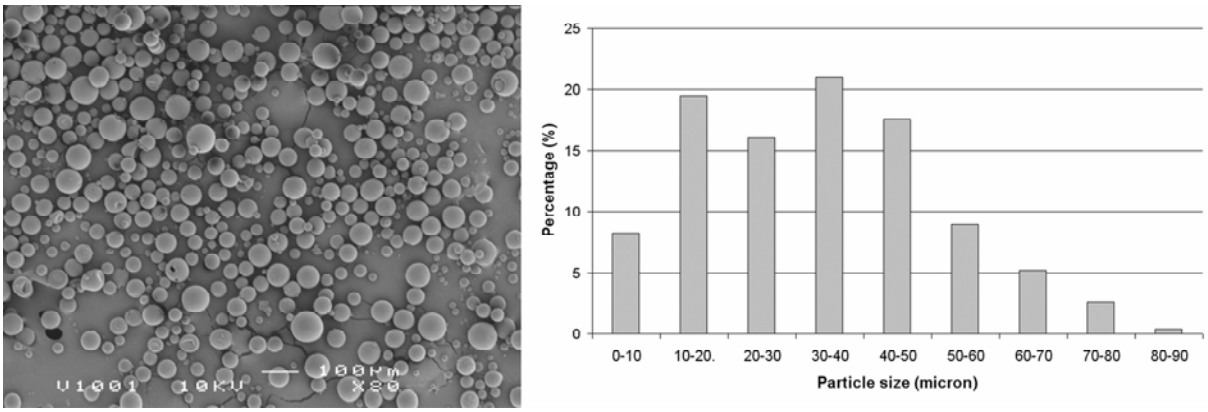



**Figure 2.** Scanning electron micrograph of the PLGA microspheres prepared by the upscaled double-emulsion technique (original magnification 80x) and size distribution graph of the PLGA microspheres (group size = 268)

### *3.2. Injectability*

In Figure 3 injectability graphs of different cement formulations are given. The force, subjected to the syringe, is expressed as a function of time. Injection time is the time span in which the paste can be injected after mixing the cement with the liquid component. This characteristic can be determined while after a certain time point pressure increases which corresponds to the hardening of the cement in the syringe. The left figure shows that with an increasing percentage of hardener in the liquid phase injection time decreased from 110 s (0.5%) to 90 s (1.0%) and 60 s (2.0%) for the microporous cement.

For the injectability tests of the PLGA/CaP samples a percentage of hardener was chosen of 1.0%. When adding PLGA microspheres to the cement, the injection time decreased from 90 s to about 75 s (10/90) and 60 s (20/80). Also the force experienced during application of the paste was higher when more microspheres were added.

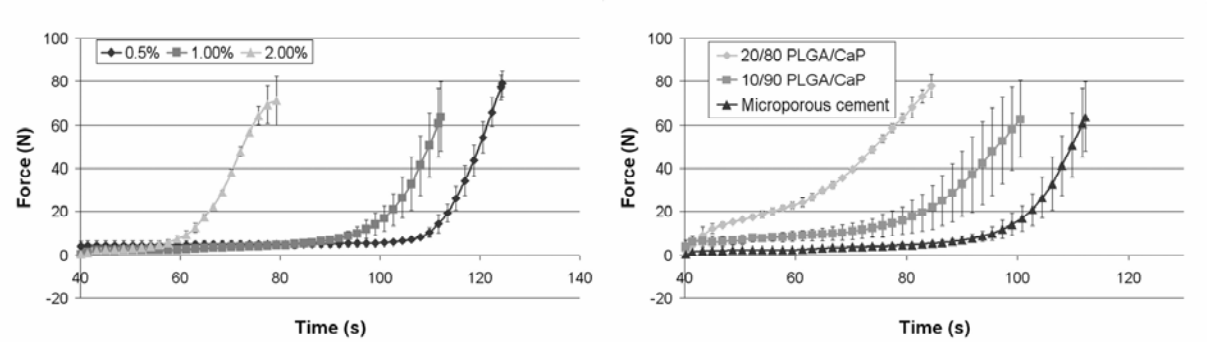



**Figure 3.** Injectability graphs of microporous cement at 0.5 % $Na_2HPO_4$ (Δ), 1% $Na_2HPO_4$ (□), 2 % $Na_2HPO_4$ (◊) (left) and of microporous cement (Δ), 10/90 PLGA/CaP(□),20/80 PLGA/CaP(◊) at 1% $Na_2HPO_4$.(right)

### *3.3. Setting time and cohesion*

Figure 4 shows the initial and final setting time of the different pastes. When microspheres were added to the CaP cement, initial setting time increased slightly but significant in both cases. Final setting time showed a non-significant increase with the 10/90 formulation and a significant increase with the 20/80 formulation.

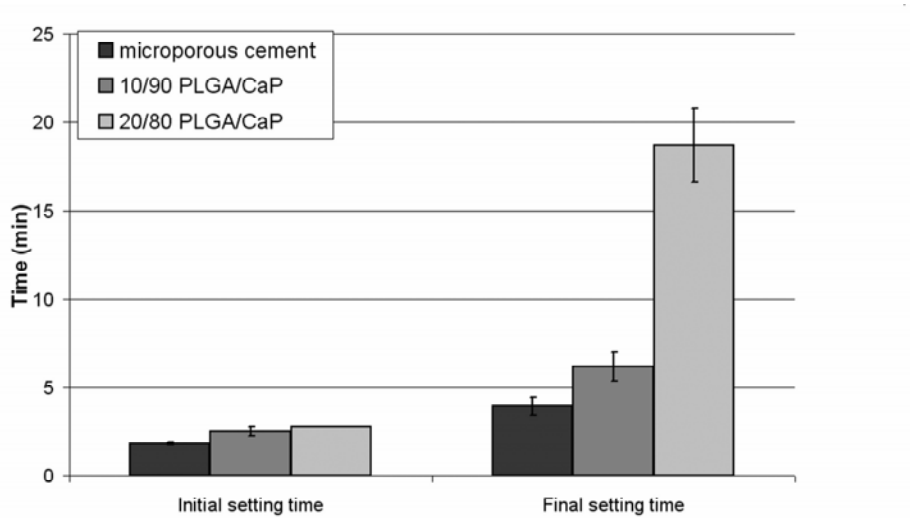



**Figure 4.** Initial and final setting time of microporous cement, 10/90 PLGA/CaP and 20/80 PLGA/CaP

The result of the cohesion test is given in Table 2. After injecting the paste in Ringer's solution the cement stayed in the desired shape during setting in all cases. In one case, using the 20/80 formulation, emission of cement particles was observed during injection.

**Table 2.** Cohesion properties of the different cement formulations

| | Microporous cement | 10/90 PLGA/CaP | 20/80 PLGA/CaP |
|---|---|---|---|
| Cohesion | ++ | ++ | + |
| Comments | - | - | Little amounts of dust after injection |

### *3.4. Porosity*

In Table 3 the porosity and macroporosity of the samples is given. The microporous CaP samples had a porosity of 41%. With the PLGA/CaP samples a higher porosity of 60% (10/90) and 69% (20/80) was obtained. The macro porosity that was introduced by the PLGA microspheres reached to 33% (10/90) and 48% (20/80) respectively.

**Table 3.** Results porosity measurement

| | Average Mass (g) | Density (g/cm$^3$) | Porosity (%) | Macro porosity (%) |
|---|---|---|---|---|
| Microporous cement | 0.2627±0.0061 | 1.835±0.039 | 40.80±1.26 | - |
| 10/90 PLGA/CaP | 0.1766±0.0029 | 1.234±0.019 | 60.20±0.53 | 32.77±1.88 |
| 20/80 PLGA/CaP | 0.1362±0.0019 | 0.951±0.012 | 69.31±0.39 | 48.18±1.38 |

### *3.5. Degradation study*

pH measurements

The left graph of Figure 5 shows the pH of the PBS solution in which the samples were situated. After week 4 significant differences were observed. The 20/80 formulation showed the highest decrease in pH, reaching to 4.0 at week 12. The same trend was seen with the 10/90 samples reaching to a pH of 4.4 and the microporous CaP samples reaching an ultimate pH of 5.5.

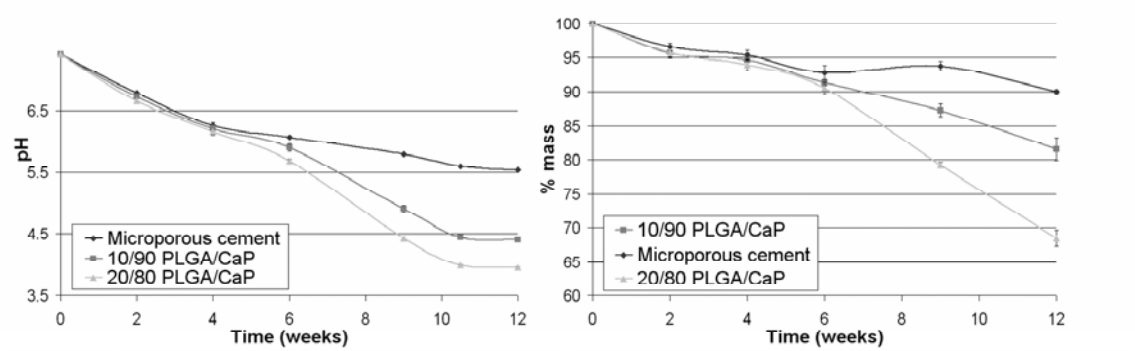



**Figure 5.** pH of the surrounding PBS (left) and percentage of initial mass (right) of the microporous cement, 10/90 PLGA/CaP and 20/80 PLGA/CaP as a function of the degradation time

Mass loss quantification

The development of the mass loss in time of the samples is given in the right graph of Figure 5. The microporous CaP as well as the other cement formulations, showed a mass loss of 5% at week 2 and 4. At week 6 a significant difference was measured between the microporous cement and the 20/80 formulation indicating PLGA erosion. Week 9 and 12 showed significant differences between all the samples, with the microporous cement remaining at 90% of its initial mass, the 10/90 samples decreasing to 82% and the 20/80 samples decreasing to 68%. Using Equation 4 the decrease in mass of the PLGA polymer inside the samples was derived (Figure 6, left). In this graph little mass loss was visible in week 2 and 4. Week 6 showed an average mass loss of 18%, which in the case of the 20/80

formulation was significantly higher than week 2 and 4. Week 9 showed a significant polymer loss reaching to 75-80%. At week 12 almost all of the PLGA had disappeared and surpassed 100% with the 20/80 formulation. No significant difference in polymer erosion rate was observed between the 10/90 and 20/80 samples.

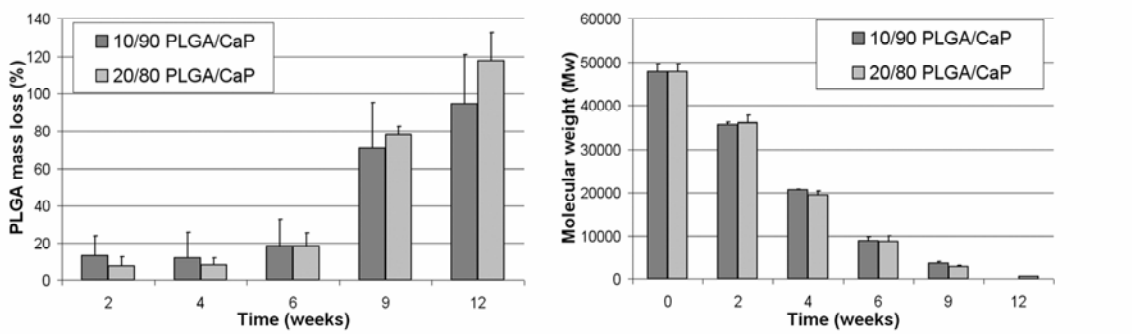



**Figure 6.** Percentage mas loss using Equation 4 (left) and weight average molecular weight ($M_w$)(right) of the 10/90 and 20/80 PLGA/CaP samples as a function of the degradation time.

Molecular weight

The $M_w$ of the PLGA polymer inside the 10/90 and 20/80 samples as a function of the degradation time is given in the right graph of Figure 6. In this graph a linear decrease of the molecular weight was observed till t=6 weeks where the $M_w$ of both samples was less than 20% of the initial value. Also a small, but significant difference was seen in $M_w$ between the 10/90 and 20/80 samples at t=9 weeks indicating faster degradation of the PLGA inside the 20/80 formulation at this time point.

Mechanical characteristics

In Figure 7 the compression strength and E-modulus of the samples is given as a function of the degradation time. At t = 3 days a significant difference in compression strength was observed between the microporous CaP and the 10/90 and 20/80 PLGA/CaP samples. When proceeding in time the strength of the microporous cement remained constant while the compression strength of the microsphere containing samples decreased significantly to 12.2 MPa (10/90) and 4.3 MPa (20/80) at day 12.

With the E-modulus the same trend was observed. At day 3 there was a significant difference between the microporous cement and both microsphere containing samples. The E-modulus of the microporous cement remained unchanged over time. The same was observed with the 10/90 PLGA/CaP samples although here a small but insignificant decrease was shown at week 12. The 20/80 formulation showed a significant decrease from 1300 MPa at day 3 to 170 MPa at week 12.

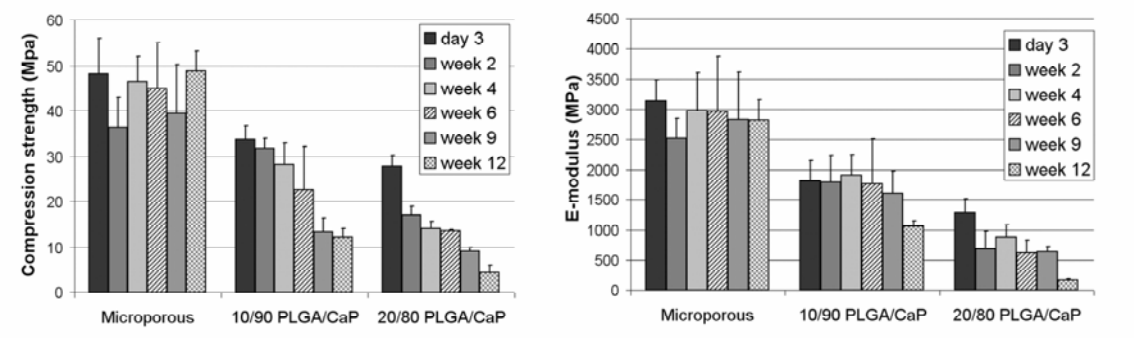



**Figure 7.** Compression strength (left) and E-modulus (right) of microporous cement, 10/90 and 20/80 PLGA/CaP samples as a function of the degradation time

Morphology

Figure 8 and 9 show SEM micrographs of the microspheres inside the 10/90 and 20/80 PLGA/CaP samples. On both samples the spheres at day 3, week 2 and 4 looked quite dense, and little erosion was observed. At week 6, bulk erosion[21] was visible resulting in flattened particles that had lost their sphericity. At week 9 the particles were almost completely eroded and thin shells of PLGA covered the inside of the holes in the cement. Finally at week 12 a structure of spherical pores was observed in which some traces of PLGA could be found. With the 20/80 formulation it was visible that the newly formed pores showed some interconnections. Also the cement showed signs of erosion at the edges of the macropores.

## 4. Discussion

In this study the applicability was investigated of PLGA microsphere/CaP cement as injectable bone filler. The mechanical and physical characteristics of the different samples as well as the degradation/erosion behaviour of the scaffolds *in vitro* was investigated and compared with a microporous CaP scaffold. Important for bone tissue engineering applications is that macroporosity is obtained during degradation, though the scaffold must retain some mechanical strength.

Regarding the mechanical/physical characteristics of the cement paste, the pattern observed for the injectability graphs showed that the addition of PLGA microspheres results in a higher stress at the tip of the syringe, increasing the initial injection pressure. The injection time also decreased with a higher amount of microspheres. The reason for this feature is that next to the cement, also the microspheres absorb some of the hardener, increasing the viscosity of the paste. In fact, for the 20/80 formulation already extra liquid phase was introduced, otherwise the paste was not injectable[22]. Consequently, the higher amount of hardener resulted in a significant higher final setting time for this formulation. Extremely important with these multiple-component cements therefore is to find an optimum L/P ratio that gives cement pastes that are injectable and in the same time have a sufficient setting time for clinical use. In addition to that, too high L/P ratio's decrease the compression strength of the cement[2].

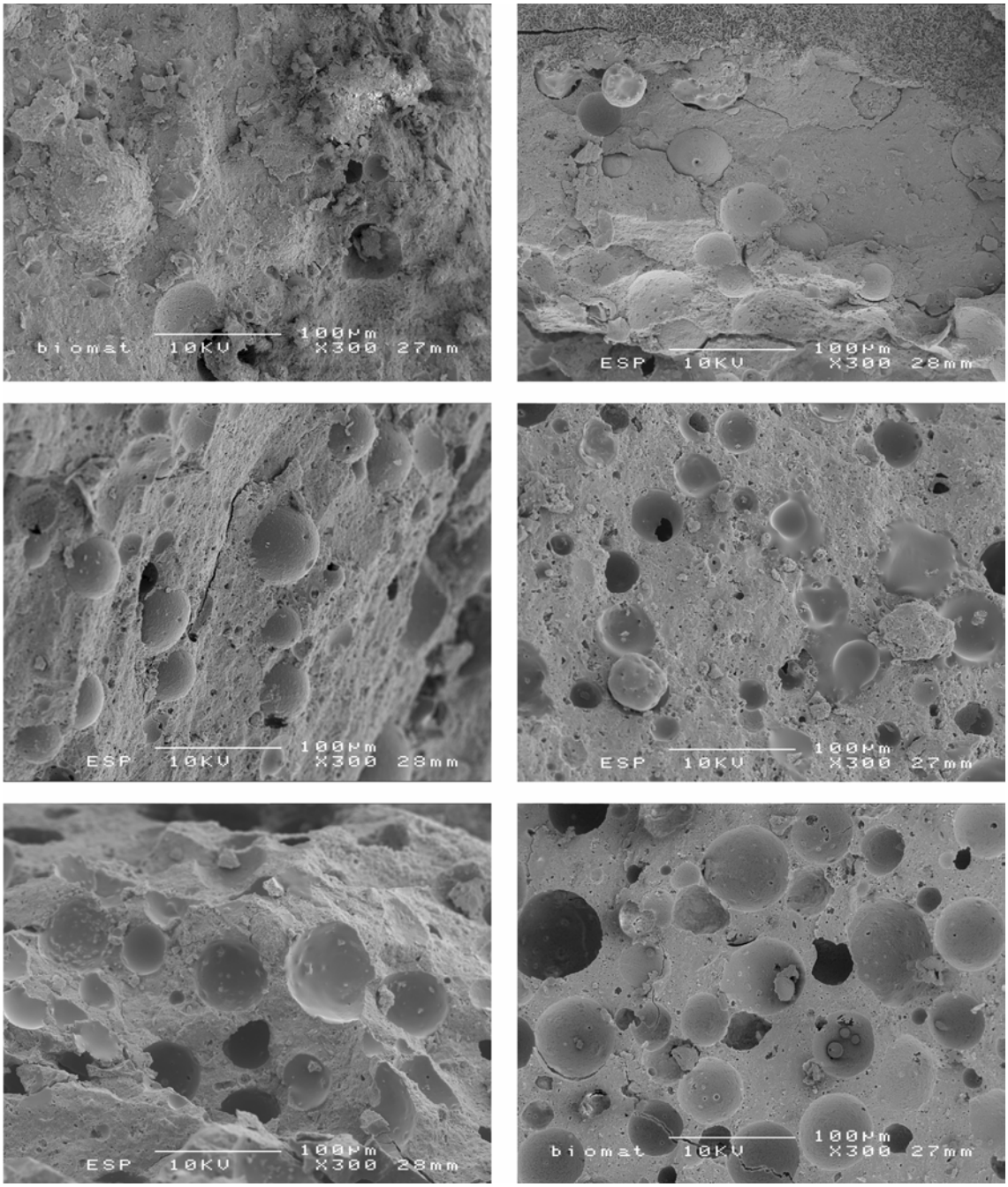



**Figure 8.** SEM-micrographs of PLGA microspheres in the 10/90 PLGA/CaP formulation: t = 3 days (upper left), t = 2 weeks (upper right), t = 4 weeks (middle left), t = 6 weeks (middle right), t = 9 weeks (lower left) and t = 12 weeks (lower right)(original magnification 300x)

Further, cement properties are influenced by the size and distribution of the microspheres. Spheres with a larger diameter overall provoke increased difficulties with clogging at the syringe opening. Consequently, in the current research relatively small microspheres were chosen. These smaller spheres have a less than optimal pore diameter for bone ingrowth as confirmed by literature[23]. Still, it has to be noticed that other more recent literature[24-25] showed already effective bone ingrowth with pore sizes starting from 50µm.

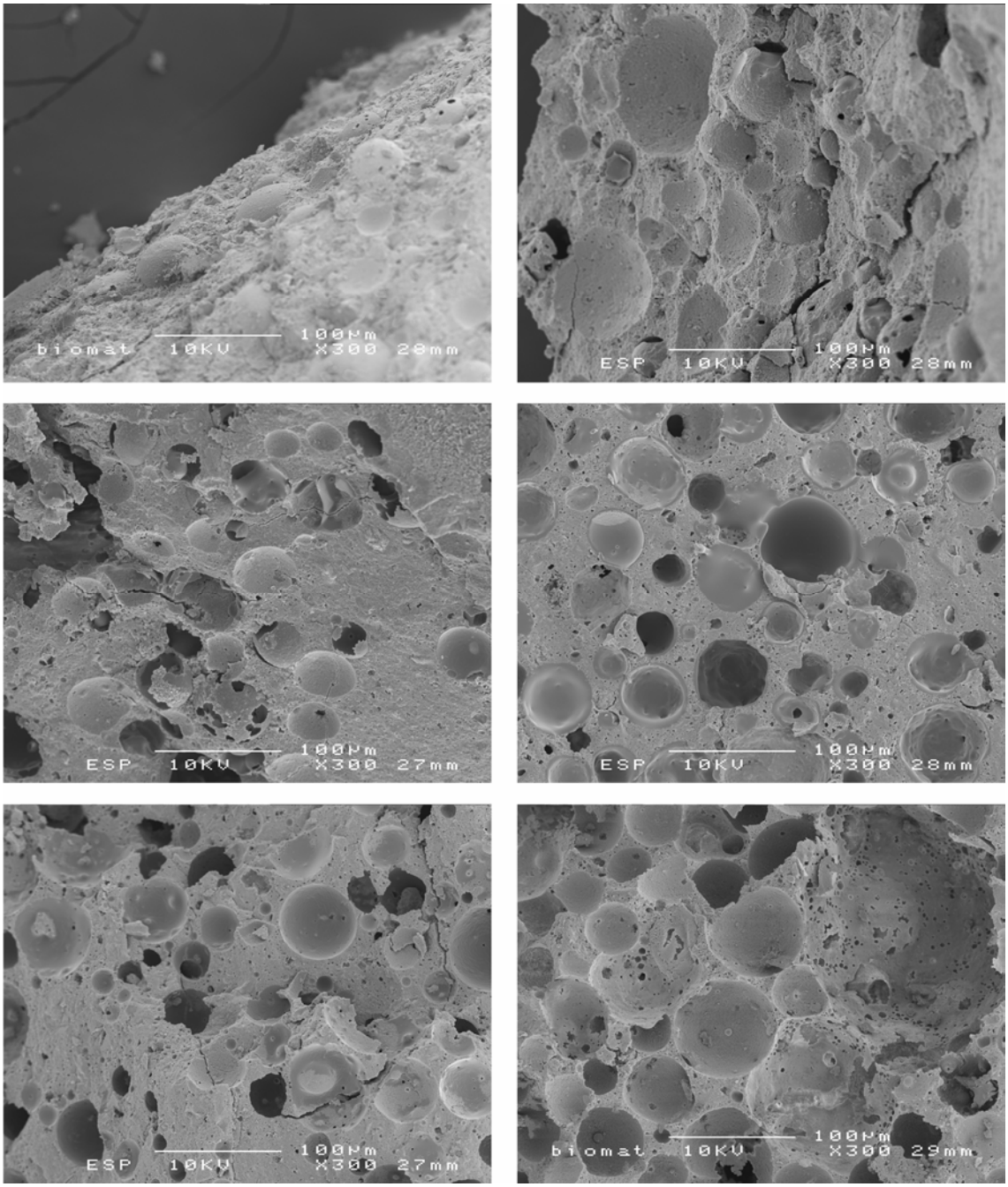



**Figure 9.** SEM-micrographs of PLGA microspheres in the 20/80 PLGA/CaP formulation: t = 3 days (upper left), t = 2 weeks (upper right), t = 4 weeks (middle left), t = 6 weeks (middle right), t = 9 weeks (lower left) and t = 12 weeks (lower right)(original magnification 300x)

In this research we used a broad particle size distribution. The particle size distribution is an important factor, while it determines the packing of the spheres. Spheres of different sizes, preferentially a bimodal distribution of larger and smaller sized spheres, can create a closer packing in which little spheres serve as interstitials between the higher sized spheres.

The porosity of the microspheres, which is determined by the (double-emulsion) process parameters[9], influences the injectability by creating more stiff or flexible spheres. The double-emulsion technique normally produces very porous structures with honeycomb morphology[17]. From cross-sections of our microspheres imbedded in epoxy-resin, the same honeycomb morphology was observed.

After cement setting, both PLGA/CaP formulations formed a scaffold in which intact PLGA microspheres were equally distributed. In literature[26] spheres in a solid matrix become interconnected when the volume fraction surpasses 0.4. According to this, the 20/80 formulation (48%) should posses an interconnective structure, while the 10/90 formulation (33%) lacks interconnectivity. SEM-micrographs of the degradation assay (Figure 8 and 9) indeed showed interconnectivity between the spheres in the 20/80 samples, while in the 10/90 samples spheres were more isolated. An open porous structure of macropores[27] was not obtained and in most cases a thin shell of HA between two neighbouring spheres could be observed by SEM. Though the presence of these shells initially forms a barrier, they are very susceptible for degradation. This is due to a local high acid concentration caused by PLGA hydrolysis and increased porosity after the PLGA has disappeared. Figure 6 gives evidence of this mechanism because PLGA erosion of the 20/80 sample surpassed 100% at t = 12 weeks. To be able to calculate the PLGA erosion with equation 4, the erosion of the CaP inside the PLGA/CaP samples was assumed to be equal to the microporous CaP samples. A PLGA erosion that surpasses 100% then automatically means that this assumption is false at this time point and that CaP erosion is higher than in the microporous cement. SEM-micrographs of this sample also showed signs of cement dissolution at these thin HA shells.

The *in vitro* degradation test showed that the overall pH of the medium of the 10/90 and 20/80 samples gave a pattern in which a significant higher decrease than the microporous CaP was observed, starting from week 6 and stabilizing around 10 weeks. This pH decrease was caused by the PLGA degradation products and exceeded the buffer capacity of the PBS buffer. A linear decrease of the pH in time was observed by Agrawal et al[28] when PLGA was mixed with HA. The graph obtained in this research showed a more exponential decrease that is similar to the the curve of normal PLGA with an overal smaller decrease in pH and a delay in onset.

Significant differences in mass loss were also visible starting from week 6 (day 42). At this time point the PLGA was already eroding, as is shown in Figure 6 (left) and is also evident in the SEM-micrographs (Figures 8 and 9). With the 10/90 and 20/80 samples it lasted more than 9 weeks and less than 12 weeks before the PLGA mass was reduced to zero. Under similar conditions complete PLGA-mass loss was observed after 8-9 weeks of degradation in literature[16,28]. The present structure in which the microspheres are surrounded by HA delays PLGA erosion due to the buffering effect of the HA and to the rigid structure of the cement that immobilizes the particles and prevents PLGA degradation products from reaching the outside medium. However, accumulations of these by-products locally decrease the pH resulting in an accelerated degradation/structural deformation of the microspheres. This result is in contrast to degradation experiments of separate spheres in which a gradual degradation is observed[29-30]. Reduction in molecular weight of the PLGA started at day 0 and was 80% at week 6 when bulk erosion was visible. A linear decrease was seen until this time point. After 6 weeks, due to erosion, less polymer was present and PLGA degradation showed a more exponential decrease.

The results observed with the compression strength assay and E-modulus showed that the PLGA microspheres provided an extra initial strength to the composite, while during/after erosion of the PLGA microspheres the compression strength significantly decreased. With the 20/80 formulation the compression strength decreased to values slightly higher than other porous cements[3-6]. The porous CaP implants by Del Real et al[6] have already shown to be biodegradable in a relative short time span (81% in 10 weeks) using a goat model. The total porosity of the implant material Del Real used was 59%. This is (slightly) lower than the porosity of the 10/90 and 20/80 samples, while the compressive strength of our samples after 12 weeks of degradation is higher (12.2 MPa and 4.3 MPa compared to 1.3 MPa). Therefore the compressive strength is most likely to be dependent on the size and rearrangement of the pores inside the cement. So the equal distribution of relatively small and polydisperse microspheres, used in this experiment, seems to be favourable with respect to the strength of the implant.

## 5. Conclusion

From the results it can be concluded that the 10/90 PLGA/CaP cement is easy to handle (injectability, initial and final setting time, cohesive properties) with the limitation that the pores left after PLGA degradation are not interconnected. The 20/80 PLGA/CaP formulation showed signs of interconnectivity, but exhibited less handling properties, though still within workable boundaries. *In vitro* PLGA degradation in both implants was comparable and lasted 9-12 weeks. The higher amount of microspheres in the 20/80 composite resulted in a higher pH decrease due to PLGA hydrolysis. Signs of CaP degradation were also found with this formulation. The compression strength of the PLGA/CaP formulations before and after 12 weeks of degradation was higher than most porous implants, though the overall porosity was comparable or even higher.

*Acknowledgements*

Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) E. Ooms, Injectable calcium phosphate cement for bone repair and implant fixation, PhD-thesis, UMC Nijmegen, department of Biomaterials, Nijmegen, The Netherlands (2003)

2) I. Khairoun, M.G. Boltong, F.C.M. Driessens, J.A. Planell, Effect of calcium carbonate on clinical compliance of apatitic calcium phosphate bone cement, J Biomed Mater Res Part A. 38 (1997) 356-360

3) H.H.K. Xu, J.B. Quinn, S. Takagi, L.C. Chow, F.C. Eichmiller, Strong and macroporous calcium phosphate cement: Effects of porosity and fiber reinforcement on mechanical properties, J Biomed Mater Res Part A. 57 (2001) 457-466

4) H.H.K Xu, C.G. Simon Jr., Fast setting calcium phosphate-chitosan scaffold: mechanical properties and biocompatibility, Biomaterials. 26 (2005) 1337-1348

5) R.P. Del Real, J.G.C. Wolke, M. Vallet-Regi, J.A. Jansen, A new method to produce macropores in calcium phosphate cements, Biomaterials. 23 (2002) 3673-3680

6) R.P. Del Real, E.M. Ooms, J.G.C. Wolke, M. Vallet-Regi, J.A. Jansen, In vivo bone response to porous calcium phosphate cement, J Biomed Mater Res Part A. 65 (2003) 30-37

7) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, rhBMP-2 release from injectable poly(DL-lactic-*co*-glycolic acid)/calcium phosphate cement composites, J Bone Joint Surg Am. 85A *Suppl* 3 (2003) 75-81

8) C.G. Simon Jr., C.A. Khatri, S.A. Wight, F.W. Wang, Preliminary report on the biocompatibility of a moldable, resorbable, composite bone graft consisting of calcium phosphate cement and poly(lactide-co-glycolide) microspheres, J Orthop Res. 20 (2002) 473-482

9) D.H.K. Kempen, L. Lu, X. Zhu, C. Kim, E. Jabbari, W.J.A. Dhert, B.L. Currier, M.J. Yaszemski, Development of biodegradable poly(propylene fumarate)/poly(lactic-co-glycolic acid) blend microspheres. I. Preparation and characterization, J Biomed Mater Res Part A. 70 (2004) 283-292

10) E.L. Hedberg, A. Tang, R.S. Crowther, D.H. Carney, A.G. Mikos, Controlled release of an osteogenic peptide from injectable biodegradable polymeric composites, J Control Release. 84(3) (2002) 137-150

11) L. Lu, G.N. Stamatas, A.G. Mikos, Controlled release of transforming growth factor beta1 from biodegradable polymer microparticles, J Biomed Mater Res Part A. 50 (2000) 440-451

12) Y.-Y. Yang, M. Shi, S.-H. Goh, S.M. Moochhala, S. Ng and J. Heller, POE/PLGA composite microspheres: formation and in-vitro behavior of double walled microspheres, J Control. Release. 88 (2003) 201-213

13) T.A. Holland, J.K.V. Tessmar, Y. Tabata, A.G. Mikos, Transforming growth factor-beta 1 release from oligo(poly(ethylene glycol) fumarate) hydrogels in conditions that model the cartilage wound healing environment, J Control Release. 94 (2004) 101-114

14) G. Fundeanu, C. Nastruzzi, A. Carpov, J. Desbrieres, M. Rinaudo, Physico-chemical characterization of Ca-alginate microparticles produced with different methods, Biomaterials. 20 (1999) 1427-1435

15) A. Gopferich, Mechanisms of polymer degradation and erosion, Biomaterials. 17 (1996) 103-114

16) K.A. Athanasiou, J.P. Schmitz, C.M. Agrawal, The effects of porosity on in vitro degradation of PLA-PGA implants used in repair of articular cartilage, Tissue Eng. 4(1) (1998) 53-63

17) G. Schwach, N. Oudry, S. Delhomme, M. Luck, H. Lindner, R. Gurny, Biodegradable microparticles for sustained release of a new GnRH antagonist—part I: Screening commercial PLGA and formulation technologies, Eur J Pharm Biopharm. 56 (2003) 327-336

18) I. Khairoun, D. Magne, O. Gauthier, J. M. Bouler, E. Aguado, G. Daculsi, P. Weiss, In vitro characterization and in vivo properties of a carbonated apatite bone cement, J Biomed Mater Res Part A 60 (2002) 633-642

19) ASTM C266, ASTM Annual Book of Standards Vol. 04.01 Cement; Lime; Gypsum, West Conshohocken, PA (1999)

20) K. Schwartz, M. Epple, A detailed characterization of poly(glycolide) prepared by solid-state polymerization reaction, Macromol Chem Phys. 200 (1999) 2221-2229

21) F. Von Burkersroda, L. Schedl, A. Gopferich, Why degradable polymers undergo surface erosion or bulk erosion, Biomaterials. 23 (2002) 4221-4231

22) M. Bohner, G. Baroud, Injectability of calcium phosphate pastes, Biomaterials. 26 (2005) 1553-1563

23) S.F. Hulbert, F.A. Young, R.S. Mathews, J.J. Klawitter, C.D. Talbert, F.H. Stelling, Potential of ceramic materials as permanently implantable skeletal prostheses, J Biomed Mater Res. 4 (1970) 433-456

24) A.I. Itälä, H.O. Ylänen, C. Ekholm, K.H. Karlsson, H.T. Aro, Pore diameter of more than 100 microm is not requisite for bone ingrowth in rabbits, J Biomed Mater Res Part A. 58 (2001) 679-683

25) J.D. Bobyn, R.M. Pilliar, H.U. Cameron, G.C. Weatherly, The optimum pore size for the fixation of porous-surfaced metal implants by the ingrowth of bone, Clin Othop Relat res. 150 (1980) 263-270

26) J. Gurland, An estimate of contact and continuity of dispersions in opaque samples, Trans Met Soc AIME. 236 (1966) 642-646

27) M. Bohner, F Baumgart, Theoretical model to determine the effects of geometrical factors on the resorption of calcium phosphate bone substitutes, Biomaterials. 25 (2004) 3569-3582

28) C.M. Agrawal, K.A. Athanasiou, Technique to control pH in vicinity of biodegrading PLA-PGA implants, J Biomed Mater Res Part A. 38 (1997) 105-114

29) X. Chen, C.P. Ooi, T.H. Lim, Effect of ganciclovir on the hydrolytic degradation of poly(lactide-co-glycolide) microspheres, J Biomater Appl. 20 (2006) 287-302

30) C. Berkland, K. Kim, D.W. Pack, PLG microspheres size controls drug release rate through several competing factors, Pharm Res. 20(7) (2003) 1055-1062

# CHAPTER 3

## PLGA MICROSPHERE/CALCIUM PHOSPHATE CEMENT COMPOSITES FOR TISSUE ENGINEERING: *IN VITRO* RELEASE AND DEGRADATION CHARACTERISTICS

*W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen*

J Biomat Sci Polym Ed, accepted december 11, 2007

## 1. Introduction

Tissue engineering is an interdisciplinary field of science in which biological substitutes are developed that are intended to restore, maintain or improve tissue function for a whole organ[1]. In general, bone tissue engineering methods apply a mechanically stable and biodegradable scaffold material containing cells (osteoblasts) and/or growth factors (bone morphogenic protein-2 (BMP-2), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF))[2-3]. Calcium phosphate cements (CPCs), though brittle, can be a suitable scaffold material for bone tissue engineering because of their osteoconductivity and perfect fit with the surrounding tissue when injected *in situ*[4]. To allow bone ingrowth into these materials, in a former experiment, macroporosity was introduced into a CPC by use of poly(lactic-*co*-glycolic acid) (PLGA) microspheres[5]. This rendered composites that had manageable handling properties and resulted, after PLGA erosion, in a highly porous scaffold.

Next to the formation of macroporosity, PLGA microspheres also can be used to obtain a controlled release of osteoinductive growth factors/drugs[6-10]. When compared to a conventional cement[11-13], introduction of these drug loaded PLGA microspheres into CPC can alter release patterns and efficiencies as well as shield the drug from chemical reactions during cement setting[14]. Ruhé et al[15-16] used PLGA microsphere CPC scaffolds for the *in vitro* and *in vivo* release of recombinant human bone morphogenic protein-2 (rhBMP-2). Main conclusion of both studies was that the introduction of drug-loaded PLGA microspheres into the cement scaffold resulted in a sustained rhBMP-2 release within 4 weeks. In the *in vivo* study[16], drug release could be tailored within certain ranges by changing the molecular weight of the PLGA and loading method. Results showed that composites where the drug was incorporated inside low molecular weight PLGA microspheres ($M_w \approx 5.000$ g/mol) revealed a higher release rate than the other groups, i.e. high molecular weight PLGA ($M_w \approx 50.000$ g/mol) and adsorption of drug onto microspheres. It was suggested that a faster degradation of the low molecular weight PLGA resulted in a higher release, and while the drug was incorporated in the PLGA less interaction with the surrounding cement occurred.

The goal of this study was to elucidate the release mechanism of a PLGA microsphere/ calcium phosphate cement composite (PLGA/CPC). Therefore, microspheres with a molecular weight of approximately 5000 g/mol were loaded with a model protein (bovine serum albumin, BSA) using two loading methods. *In vitro* BSA release from PLGA/CPC, CPC and PLGA microspheres into an aqueous solution was investigated by reversed-phase high performance liquid chromatography (RP-HPLC). Furthermore, similar to a previous performed degradation experiment[5], physical and mechanical properties of the PLGA/CPC were determined and an *in vitro* degradation study was performed in phosphate buffered saline (PBS).

## 2. Materials and Methods

### *2.1. Materials*

Poly(lactic-*co*-glycolic acid) (PLGA, $M_n$ = 2838 ± 3 g/mol, $M_w$ = 4547 ± 32 g/mol, PDI = 1.60 ± 0.01, L/G = 52/48, Purasorb®, Purac, Gorinchem, Netherlands) was used for the preparation of the microspheres. Poly(vinyl alcohol) (PVA, 88% hydrolyzed, $M_w$ = 22000 g/mol, Acros Organics, Geel, Belgium) was used as stabilizer during microsphere preparation. The calcium phosphate cement (Calcibon®, Biomet Merck, Darmstadt, Germany) consisted of 61% α-tricalcium phosphate (α-TCP), 26% $CaHPO_4$, 10% $CaCO_3$ and 3% precipitated hydroxyapatite (HA). The cement liquid was a 1% w/v $Na_2HPO_4$ in demineralized water ($ddH_2O$).

### *2.2. Methods*

#### *2.2.1. Preparation PLGA microspheres*

PLGA microspheres were prepared using a (w/o/w) double emulsion solvent evaporation technique described in a previous experiment[5]. Briefly, 1.4 g of PLGA was dissolved in 2 ml of dichloromethane (DCM) inside a 50 ml glass tube. 500µl of demineralized water ($ddH_2O$) was added to the polymer solution while vortexing vigorously for 1 min. Subsequently 6 ml of a 0.3% w/v PVA solution in $ddH_2O$ was added and vortexing was continued for another 1 min. The content of the 50 ml tube was transferred to a stirred 1000 ml beaker and another 394 ml of 0.3% w/v PVA solution was added slowly. This was directly followed by slowly adding 400 ml of a 2% v/v isopropylic alcohol (IPA) solution in $ddH_2O$. The suspension was stirred for 1 h. Spheres were allowed to settle for 15 min and the clear solution on top was decanted. Finally, spheres were washed with $ddH_2O$, freeze-dried for 24 h and stored under argon at -20°C. Morphology of the microspheres was analyzed by scanning electron microscopy (SEM) (JEOL 6400-LINK AN 10000 at 10 kV). For this, samples were mounted on aluminium stubs and sputtered with gold-palladium prior to examination. From the SEM-micrographs, average size of the microspheres was determined (n = 200) using digital image software (Leica Qwin®, Leica Microsystems AG, Wetzlar, Germany).

#### *2.2.2. Preparation PLGA/CPC*

20% w/w PLGA microsphere/CPC composite samples for the degradation or release test were made by adding 200 mg PLGA microspheres to 800 mg CPC powder inside a 2 ml plastic syringe, after which the contents were stirred vigorously for 15 s to achieve a good distribution of spheres inside the cement (Silamat® mixing apparatus, Vivadent, Schaan, Liechtenstein). Subsequently, 350 µl 1% w/v $Na_2HPO_4$ in $ddH_2O$ was added to the mixture and the contents were stirred again for 15 s. The resulting paste was injected into a Teflon mould (cylinders 4.5 x 9 mm) where it was allowed to harden at room temperature overnight.

*2.2.3. Physical/Mechanical properties PLGA/CPC*

Initial and final setting time, total/macroporosity and compression strength/E-modulus of the PLGA/CPC was determined[5]. In brief, the microsphere composites were subjected to the following analyses.

Setting time

Initial and final setting time was assessed using custom available Gillmore needles (ASTM C266). For this, a bronze block containing 6 holes (6 mm in diameter, 12 mm in height) was used as a mould and placed in a water bath at body temperature (37˚C). Samples were mixed and injected into the mould in a retrograde fashion, after which the initial and final setting time was determined. Tests were done in triplicate.

Porosity

Total- and macroporosity of preset samples was determined. Macroporosity is the porosity generated by the degradation of PLGA microspheres and corresponds to the volume percentage of microspheres initially. The total porosity is the macroporosity plus the original microporosity of the cement. For this, both PLGA/CPC samples and CPC samples of a known volume were placed in an oven at 650°C for 2 h. After burning out the PLGA/moisture, samples were weighed and equation 1 and 2 were used for the derivation of the total porosity and the macroporosity.

$$\varepsilon_{tot} = (1 - \frac{m_{macro/micro}}{V * \rho_{HAP}}) * 100\% \qquad \textit{Equation 1}$$

$$\varepsilon_{macro} = (1 - \frac{m_{macro}}{m_{micro}}) * 100\% \qquad \textit{Equation 2}$$

*Legend:* $\varepsilon_{tot}$ = total porosity (%), $\varepsilon_{macro}$ = macroporosity (%), $m_{macro}$ = average mass of macroporous sample (after burning out gelatin) (g, n = 3), $m_{micro}$ = average mass of CPC sample (g, n = 3), V = volume sample ($cm^3$), $\rho_{HAP}$ = density of hydroxyapatite ($g/cm^3$)

Mechanical characteristics

Preset PLGA/CPC samples were soaked in Ringer's solution for 3 days. Thereafter, they were placed in a tensile bench (858 MiniBionixII®, MTS Corp., Eden Prairie, MN, USA) and compression strength and E-modulus along the height of the specimens was measured at 0.5 mm/min crosshead speed.

*2.2.4. Degradation study*

For the degradation study, preset PLGA/CPC and CPC samples were placed in 3 ml phosphate buffered saline (PBS, pH = 7.4) and incubated at 37˚C in a water bath on a shaker table (70 rpm) for 12 weeks. Sample medium was refreshed every week and 5 specimens were subjected to analysis at day 14, 28, 42, 63 and 84 of incubation.

pH measurement

Directly after removal of the samples from the water bath, pH of the PBS medium was measured.

Mass loss quantification

The samples were vacuum dried overnight before measuring the mass. Mass change of the samples was calculated using Equation 3.

$$R_C = \frac{m_0 - m_n}{m_0} * 100\% \qquad \textit{Equation 3}$$

*Legend:* $R_C$ = mass change of sample on t = n (%), $m_0$ = mass sample on t = 0 (g), $m_n$ = mass sample on t = n (g)

Molecular weight analysis

The molecular weight of the PLGA polymer inside the PLGA/CPC was monitored using gel permeation chromatography (GPC). The system comprises of a L2130 HPLC pump, a L-2400 UV detector and a L-2490 RI Detector (Hitachi corp., Tokyo, JPN). The GPC column used was a 4.6*300 mm Styragel® HR 4E column with a 4.6*30 mm Styragel® guard column (Waters Corp., Milford, MA, USA). Tetrahydrofuran (THF) was applied as eluent at a flow rate of 0.35 ml/min. Polystyrene standards (SM-105, Shodex corp., Tokyo, JPN) were used to obtain a primary calibration curve. For the calculation of the number average molecular weight ($M_n$) or weight average molecular weight ($M_w$) of the PLGA, Mark-Houwink constants of PLGA 50:50 (a = 0.761, K = $1.07*10^{-2}$, THF, 22.5°C)[17] and PS (a = 0.717, K = $1.17*10^{-2}$, THF, 25°C) were used. To extract the PLGA, 100 mg of the samples was grinded and put in a sample flask together with 3 ml of THF. The suspension was put on a shaker table for 2 h and was purified using 13 mm GHP 0.2µm Acrodisk® filters (Waters).

Mechanical characteristics

Compression strength of the samples after incubation was measured using a tensile bench as described in "Physical/Mechanical properties PLGA/CPC".

Morphology

Morphology of the different cement formulations was analyzed using SEM. Degradation behavior of the PLGA microspheres was visualized at a magnification of 250x.

*2.2.4. BSA Release test*

Preparation BSA loaded microspheres and PLGA/CPC

PLGA/CPC as well as PLGA microspheres and CPC controls were loaded with bovine serum albumin (BSA) as presented in Table 1. With CPC, 20 µl of a 8.0% w/v BSA solution in $ddH_2O$ was added and disks were dried at room temperature. With the PLGA/CPC and PLGA microspheres, BSA was incorporated (BSA(I)) into as well as adsorbed (BSA(A)) onto the microspheres[16]. For the BSA(I) samples, a BSA solution (11.2% w/v BSA in $ddH_2O$) was added to the initial water phase during microsphere preparation. The BSA(A) samples were prepared by adding 800µl of a 5.0% w/v BSA solution in $ddH_2O$ to 1.0 g of microspheres, after which the BSA was freeze-dried onto the spheres. After loading the spheres, PLGA/CPC was prepared as described in "Preparation PLGA/CPC". Entrapment efficiency of the microspheres was determined by dissolving a known amount of spheres

in sodium hydroxide (NaOH) after which the solution was neutralized with hydrochloric acid (HCl). BSA concentration in the resulting solution was measured by high performance liquid chromatography (HPLC) using a reversed-phase (RP)-HPLC column (Atlantis®, Waters corp., Milford, MA, USA) and the same HPLC system as was applied with GPC with the UV detector at 280nm. A 40/60 mixture of acetonitril/water was used as mobile phase containing 10% v/v 0.1M formic acid. Before each analysis samples were filtered using Acrodisk® filters (Waters).

**Table 1.** Loading of BSA per implant

| | **Sample dimensions** | **Theoretical loading (mg)** | **Entrapment efficiency (EE,%)** | **Actual Loading (mg)** |
|---|---|---|---|---|
| **CPC** | 7.7*2.0 | 1.6 | - | - |
| **PLGA/CPC** | (radius*height,mm) | | | |
| BSA (A) | 4.5*9.0 | 1.5 | - | - |
| BSA (I) | 4.5*9.0 | 1.5 | - | - |
| **PLGA Microspheres** | (amount, mg) | | | |
| BSA (A) | 37 | 1.5 | 85.7 ± 2.8 | 1.29 ± 0.04 |
| BSA (I) | 37 | 1.5 | 73.4 ± 4.1 | 1.10 ± 0.06 |

Release assay

During the release test, 3 samples of each formulation were incubated in demineralized water ($ddH_2O$). Initially, 1.6 ml of $ddH_2O$ was added after which samples of 200 µl were taken at t = 0.5, 1, 2 and 24 h. Thereafter, the sample medium was kept at 1 ml and samples were taken at t = 2, 3, 4, 7 days and accordingly every week until 42 days. Starting from 1 day, sample medium was refreshed every time a sample was taken. Samples were analyzed by RP-HPLC, and taken in triplicate (n=3).

*2.2.5. Statistical analysis*

Data were presented as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat® (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

### *3.1. Preparation microspheres/cement*

The prepared PLGA microspheres showed a slightly deformed spherical structure after freeze-drying as is visible in Figure 1. Also some empty microsphere shells were observed as well as the inclusion of smaller microspheres inside larger ones. The average size of the microspheres was 28.67 µm (Table 2). Cement properties of the PLGA/CPC showed a total porosity of 75.1% and macroporosity of 57.1%. Compression strength (11.6 MPa) was markedly lower than the CPC. The PLGA/CPC also demonstrated a higher initial setting time than the CPC ($p<0.001$). Final setting time of the PLGA/CPC was longer than 20 min, though was reached within 24 h.

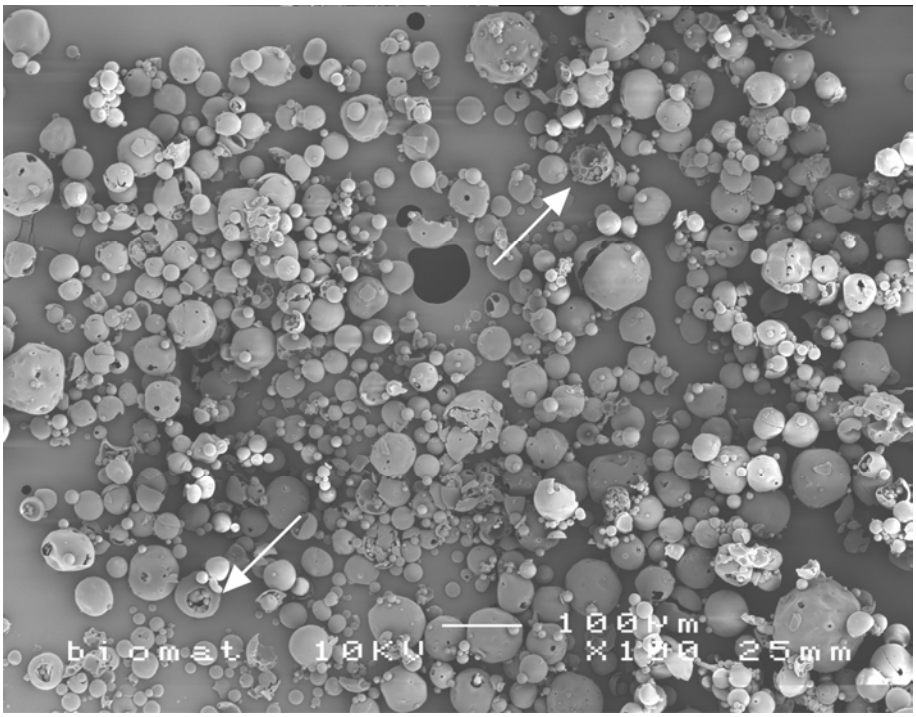



**Figure 1.** SEM-micrograph of PLGA microspheres after freeze-drying where the arrowheads point out inclusion of small spheres into larger ones (original magnification 100x)

**Table 2.** Characteristics of PLGA/CPC and CPC

| | PLGA/CPC | CPC |
|---|---|---|
| Particle size (µm) | 28.67 ± 25.65 | - |
| Sample weight (mg) | 176.4 ± 9.0 | 262.7 ± 6.1 |
| Porosity (%) | 75.1 ± 2.2 | 40.8 ± 1.3 |
| Macroporosity (%) | 57.9 ± 2.0 | 0 |
| Compression strength (MPa) | 11.6 ± 4.7 | 64.4 ± 16.6 |
| E-modulus (MPa) | 748.9 ± 239.0 | 5435.6 ± 1331.9 |
| Initial setting time (s) | 210 ± 0 | 108 ± 6 |
| Final setting time (s) | > 1200* | 265 ± 31 |

* Final setting time reached within 24 h

### *3.2. Degradation test*

The results of the pH test are given in Figure 2. The medium of the PLGA/CPC showed a fast pH decrease in the beginning but stabilized after 42 days at a pH of 4.5. This was significantly lower than the CPC control where pH stabilized at 5.8. The development of the mass of the PLGA/CPC and CPC is given in Figure 3. The PLGA/CPC demonstrated a significant decrease in mass ($p<0.01$) already after 14 days, whereas the CPC samples showed a 2% increase in mass. The mass of the PLGA/CPC decreased further to a total loss of 22%, slightly higher than the weight % of microspheres inside the composite. The molecular weight of the PLGA microspheres (Figure 4) showed a significant decrease to $M_n \approx 1800$ g/mol, $M_w \approx 2300$ g/mol after 28 days incubation time. At t = 42 days and at later time points the PLGA was completely degraded. Compression strength of the samples (Figure 5) also revealed a significant decrease from 13.8 MPa at t = 3 days to 4.6 MPa after 84 days. SEM-micrographs of the PLGA/CPC at the different time points are shown in Figure 6. Directly after mixing, few microspheres were visible and the cement appeared to be dense. At 14 days, a structure of

degraded polymer inside microsphere cavities was observed. No PLGA polymer was observed after 4 weeks and small, spherical structures were present inside the pores. Furthermore, after 63 and 84 days the calcium phosphate matrix showed a structural deformation and pores became less spherical.

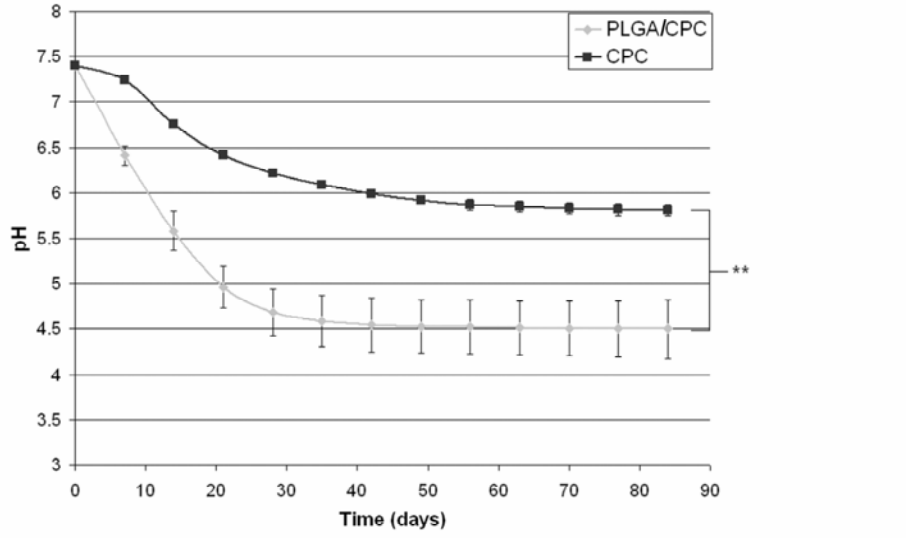



**Figure 2.** pH change of the medium of the PLGA/CPC and CPC during the degradation test, ** = p<0.01

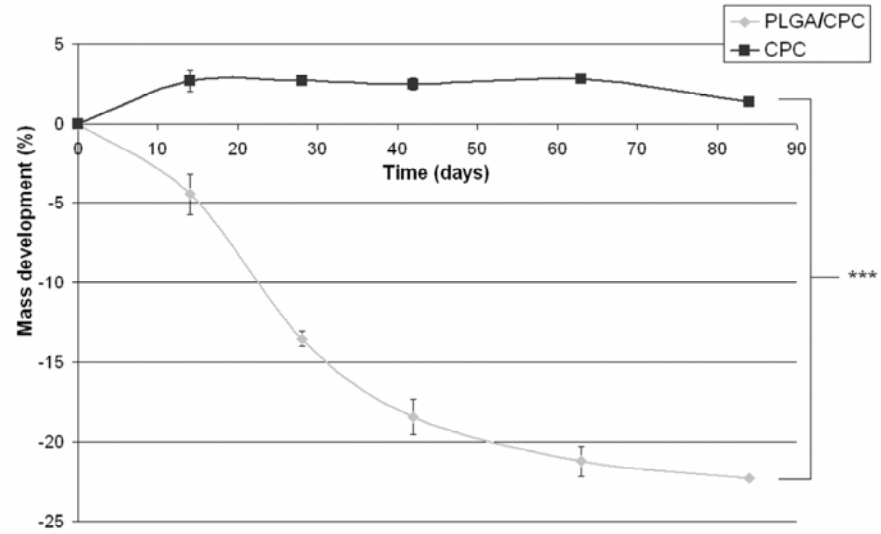



**Figure 3.** Mass development of the PLGA/CPC and CPC during the degradation test, *** = p<0.001

### *3.3. Release test*

Entrapment efficiency of the microspheres is given in Table 1. Efficiency of the PLGA microspheres reached 87% with the BSA(A) samples, whereas with the BSA(I) microspheres (73%) it was significantly lower ($p<0.05$). Entrapment efficiency of the PLGA/CPC and CPC could not be measured.

Figure 7 depicts the result of the release assay with the PLGA/CPC, microspheres and CPC control. All PLGA microspheres exhibited a high burst release, but compared to the BSA(A) microspheres, inclusion of the BSA (BSA(I)) resulted in a lower burst. With both groups a small sustained release up to 42 days was present that was slightly enhanced with the BSA(I) microspheres. When expressed as percentage of actual loaded BSA, release efficiency after 42 days reached approximately 80% with the

BSA(I) groups and 100% with the BSA(A) groups. The release patterns of the PLGA/CPC or CPC samples were different from the PLGA microspheres.

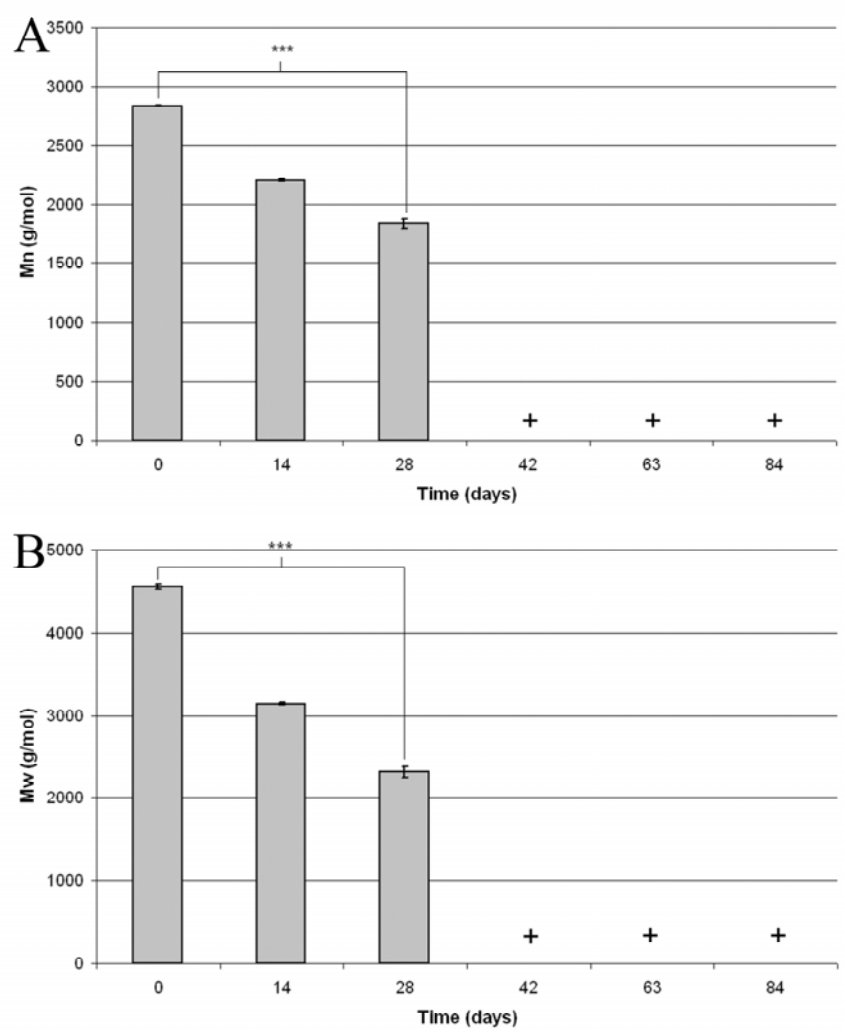



**Figure 4.** Molecular weight of PLGA during the degradation test, A = Number average molecular weight ($M_n$), B = weight average molecular weight ($M_w$), ***= $p<0.001$ (+=fully degraded PLGA)

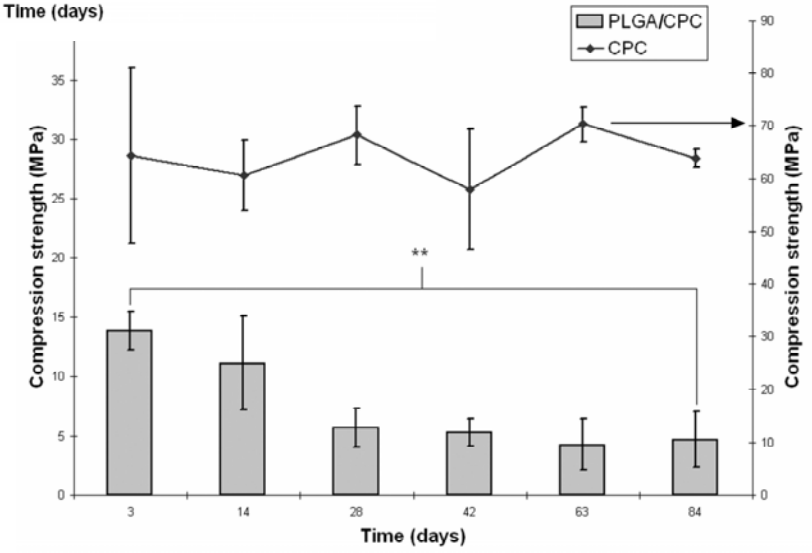



**Figure 5.** Compression strength of the PLGA/CPC and CPC during the degradation test, **=$p<0.01$

Within all groups the total amount of BSA released after 42 days was less than 20%, and virtually no burst release was obtained. The PLGA/CPC samples overall followed a similar release pattern as the CPC, and showed a release of 4.5-6.4% within the first week followed by virtually no sustained BSA

release. With the BSA(A) samples, release after 7 days even was significantly less ($p<0.05$) than the CPC.

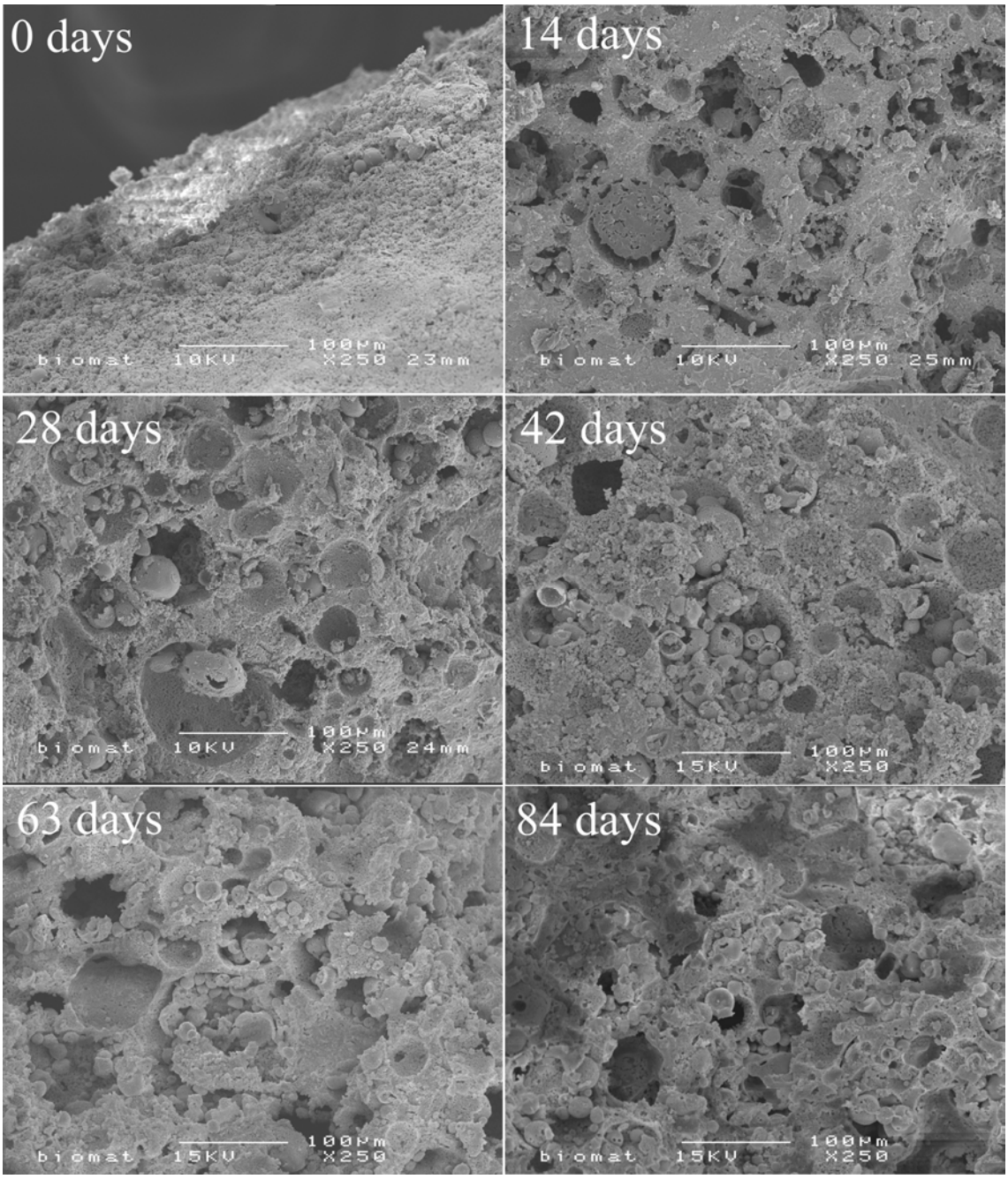



**Figure 6.** SEM-micrographs of the PLGA/CPC during the degradation test (original magnification 250x)

## 4. Discussion

To elucidate the release mechanism of PLGA microsphere/CaP cement composites, in this study a low molecular weight PLGA/CPC was investigated for *in vitro* degradation and drug release characteristics using BSA as a model protein. Because drug release from PLGA/CPCs is hypothesized to be the result of PLGA microsphere degradation, both degradation and release data should be investigated.

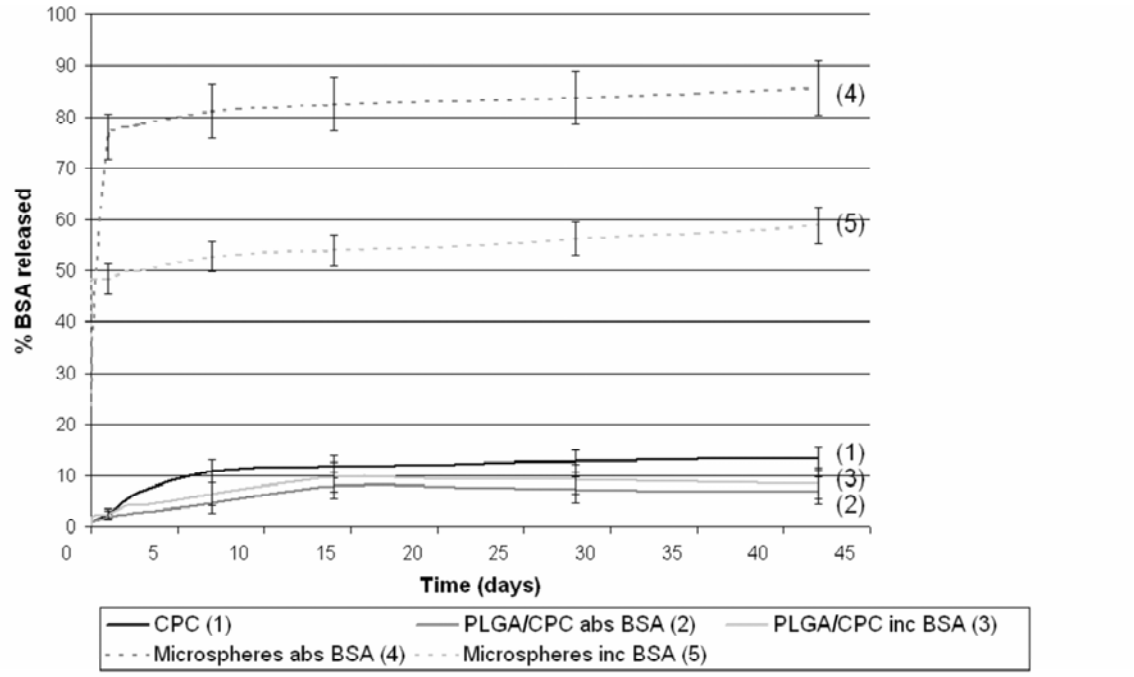



**Figure 7.** BSA release from the PLGA microspheres, PLGA/CPC and CPC expressed as percentage (%) of the theoretical loaded BSA

Degradation characteristics and mechanical/physical properties of the PLGA/CPC were determined according to an earlier performed study with higher molecular weight PLGA microspheres ($M_w$ = 50.000 g/mol)[5]. *In vitro* release characteristics were assessed by measuring the concentration of BSA in the release medium by RP-HPLC. Loading was performed by inclusion of BSA inside the microspheres during the double emulsion process or adsorption of BSA onto the microspheres by freeze-drying. BSA-loaded CPC and PLGA microspheres were taken as reference materials.

Results from the degradation test showed a fast degradation of the PLGA microspheres, which resulted in a structure of spherical and interconnected pores inside the cement already after 4 weeks. Because of this fast degradation, also the pH (hydrolysis) and compression strength decreased quite dramatically. The CPC showed a much smaller pH decrease that was caused by cement reorganization into apatite or dissolution of one of the cement starting components (monetite)[18]. Compared to a PLGA/CPC with PLGA of $M_w \approx 50.000$ g/mol[5], degradation was significantly enhanced. Degradation rates of these composites therefore can be increased by decreasing the molecular weight of the PLGA. Improved degradation of the PLGA microspheres can be beneficial for *in vivo* conditions but the pH decrease should not induce inflammation[19] and mechanical stability should be sufficient to provide the porous structure.

Besides fast microsphere degradation, also signs of cement degradation were observed. Results of the PLGA/CPC showed a higher mass loss than the percentage of microspheres added initially, whereas the CPC only showed an increase in mass that was caused by precipitate from the PBS solution[20]. SEM pictures of the samples showed loss of structural stability after 9 and 12 weeks that could be related to cement degradation and an increase in the microporosity of the cement. Explanations for

improved cement degradation are acidic dissolution by PLGA hydrolysis or the higher porosity of the composites after PLGA degradation. SEM investigation revealed also the formation of spherical structures inside the macropores. These structures were calcium phosphate shells that precipitated onto small PLGA microspheres, which were trapped inside the original microspheres. Such a microsphere structure was seen with SEM (Fig.1) and is proof of the formation of microcapsules[21] which also involves the encapsulation of smaller spheres by larger ones during the double emulsion technique as a result of suboptimal process parameters. These process parameters also explain the difference in entrapment efficiency that was observed for BSA(I) (73%) and BSA(A) (87%) microspheres. During double-emulsion, BSA can diffuse through the microsphere shell to the surrounding medium resulting in decreased entrapment efficiencies[22]. Unfortunately, entrapment efficiencies of the PLGA/CPC and CPC controls could not be determined using the same method as a result of electrostatic interactions between the BSA and the cement.

From the *in vitro* release test, multiple observations were made. First of all, the introduction of PLGA microspheres inside the cement minimized the burst release that was observed with separate PLGA microspheres. Burst release from the PLGA microspheres occurs when loosely bonded proteins near or onto the surface dissolve directly after exposing them to the medium. With the BSA(A) microspheres, almost all of the BSA was released within 1 day, indicating that freeze-drying the BSA onto the spheres does not result in a strong physical bond. Furthermore, a small sustained release with both BSA(A) and BSA(I) microspheres was observed. This sustained release is a result of the degradation of the microspheres or, with the BSA(I) samples, diffusion of the BSA through the particle surface[23]. The release curve that was observed with the PLGA/CPC was quite different from the separate microspheres and comparable to the CPC. It consisted of a low initial release within 1 week that was followed by a period of no release. The introduction of PLGA microspheres in these formulations therefore did not markedly improve the release efficiency or altered the release pattern from the cement. A plausible explanation for the release pattern observed for both PLGA/CPCs is binding of BSA to the cement after release from the (degraded) PLGA microspheres. Furthermore, during cement mixing loosely bonded BSA can dissolve into the liquid hardener thereby distributing the BSA evenly through the cement.

With the BSA(A) PLGA/CPC initial release was even less than the CPC control. From the degradation experiment we observed that compared to CPC, with PLGA/CPC a stronger pH decrease occurred within the first weeks as a result of microsphere degradation. Such a pH changes the state of the cement surface, which in the release study resulted in a stronger electrostatic bond with the BSA. In more detail, physical bonds between the cement and proteins occur because the surface of the cement is covered by negatively charged phosphate groups and positively charged calcium groups. The state of the phosphate groups is pH dependent, and can shift from $HPO_4^{2-}$ to $H_2PO_4^-$ when pH decreases, thereby changing the overall charge of the cement surface. For HA, the point of zero charge (pzc), comparable to the isoelectric point of proteins, lies around pH = 6.5-7.3[24-25]. This indicates that at an

acidic pH the surface of HA, the end product of our cement, is positively charged. Negatively charged proteins, like BSA (pI = 5.0), therefore will experience a stronger physical attraction with the cement when pH decreases from a neutral to a mild acidic pH as was present initially with the PLGA/CPC but also in a lesser extent with the CPC. This phenomenon was also described by Hughes Wassell et al[26], who observed a 1.3 times increase of adsorbed BSA onto HA powder when pH dropped from 7.15 to 5.15. We performed a similar experiment with our cement where BSA loaded microspheres and cement powder were added to 3 ml of medium at pH =7.4 (PBS) and medium of pH = 3.35 (citric acid). Results after 1 day are listed in Table 3 and show an approximately 3 times decrease in %BSA released with the acidic buffer for both BSA(A) and BSA(I) microspheres, indicating a strong pH dependency. Alternatively, basic proteins can experience a weaker binding with the cement at an acidic pH. This mechanism explains the slightly improved *in vitro* BMP-2 (pI =8.5) release from a PLGA/CPC in acidic buffer (pH = 4.0) that was observed by Ruhé et al[15].

In an *in vivo* situation, local pH inside a calcium phosphate implant can also decrease leading to a similar phenomenon. Overall, *in vivo* BMP-2 release from a comparable PLGA/CPC[16] showed a better release efficiency and higher sustained release after 4 weeks than an *in vitro* BMP-2 release study using the same composites[15]. Under the *in vivo* conditions, absorption of proteins from the surrounding body fluid will influence drug release as they can compete with protein binding sites at the cement interface. Alternatively, a higher volume of the body fluid and/or a better flow inside the composites neutralizes the acidic degradation products more effectively.

After PLGA microsphere degradation, the amount of acidic degradation products decreases. As the state of the cement surface changes back in neutral conditions, it is hypothesized that adsorbed proteins like BSA will experience a weaker interaction with the cement, resulting in a delayed release. Also the increase in cement surface area after microsphere degradation can improve drug release from the cement. Such a release pattern can explain the enhanced release that was observed with lower molecular weight PLGA/CPC ($M_w \approx 5.000$) in the earlier described 4 weeks *in vivo* release study by Ruhé et al[16]. Within this time period these microspheres should be completely degraded whereas the higher molecular weight PLGA microspheres ($M_w \approx 50.000$) are still present[5]. With our *in vitro* release study we did not observe this feature after 6 weeks, which could be the result of the small volume and low buffering capacity of the medium.

Next to the release pattern, structural integrity of the drug is also an important issue as the drug should be active upon release into the surrounding medium. When drugs are released from PLGA microspheres, in most cases there is some evidence of structure loss and degradation[27-29]. Even during the encapsulation of drugs in the w/o/w double-emulsion process, loss of tertiary structure and degradation occurs[30]. Furthermore, if drug is dispersed into the cement upon mixing, the harsh chemical conditions during cement setting possibly can degrade it[14]. The end product of our cement, hydroxyapatite(HA), is not likely to harm the protein structure as it is known for its stabilizing effect on drugs/proteins[31]. However, in literature[32], structural degradation of growth factors like BMP-4

and bFGF is observed after release from various ceramic scaffolds. Unlike previous studies performed with $^{125}$I-labelled growth factor[16], the used analysis method (RP-HPLC) in our study gives some information about the structure and stability of the released BSA. However, to get a more precise impression of the BSA structure, i.e. molecular weight, in our release experiment a pilot study was performed in which sample medium of the PLGA/CPC and microspheres at different time points was investigated by sodium dodecyl sulphate poly(acryl amide) gel electroforesis (SDS-PAGE)[32] and matrix assisted laser dissorption ionization-time of flight mass spectrometry (MALDI-TOF MS)[33]. Corresponding to the high burst in the release study, results from SDS-PAGE (Figure 8) showed a strong signal at the height of BSA for the PLGA microspheres at day 1 of incubation whereas no signal was measured with the PLGA/CPC. Further investigation of the release medium of the PLGA/CPC with MALDI-TOF MS (Figure 9) showed some peaks in the range 2,000-14,000 g/mol with the sample that was taken after 1 week, whereas after 4 weeks almost no signal was observed. Degradation of the BSA therefore seems to occur for the PLGA/CPC, but only a very low concentration of these degradation products was found.

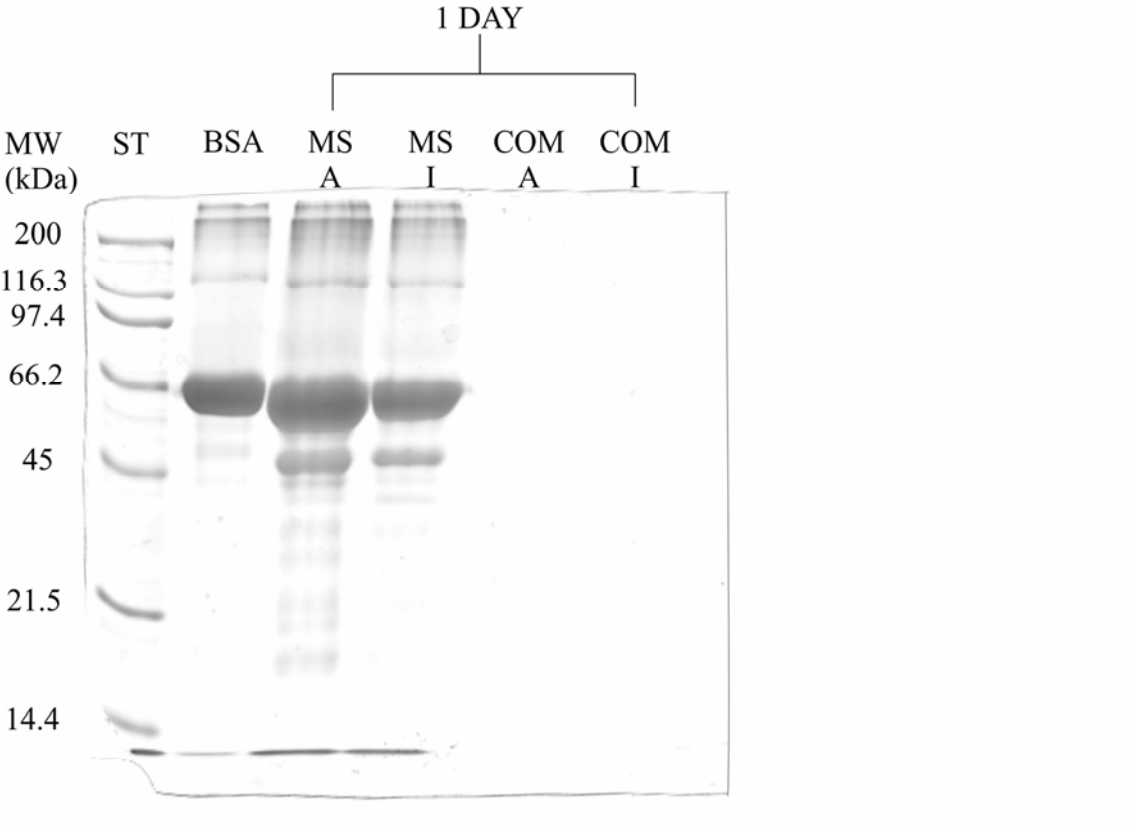



**Figure 8.** SDS-PAGE chromatogram of BSA released from PLGA microspheres and PLGA/CPC after 1 day incubation in release medium; ST=standard, MS = microspheres, COM = PLGA/CPC composites, A = BSA adsorbed onto PLGA microspheres, I = BSA incorporated into PLGA microspheres

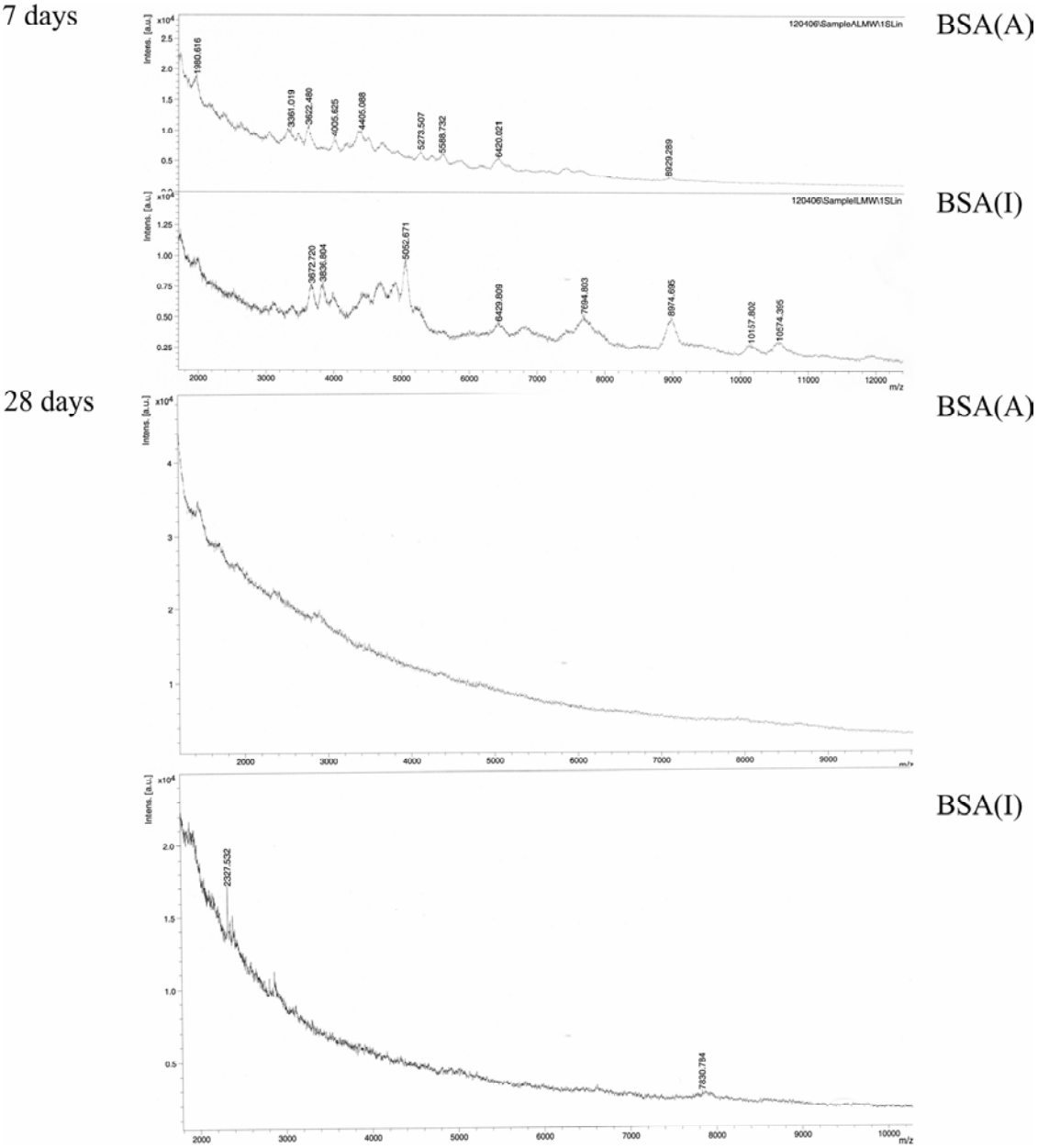



**Figure 9.** MALDI-TOF MS spectrum within a molecular weight range of 2000-10000 g/mol featuring products released from the PLGA/CPC after 7 and 28 days of incubation in release medium

## 5. Conclusion

By changing the molecular weight of the PLGA, *in situ* degradation rates of incorporated microspheres and concomitant formation of macroporosity can be tailored. *In vitro* BSA release until 6 weeks from PLGA/CPC was marginal and not improved when compared to BSA loaded CPC. Separate PLGA microspheres showed high release efficiencies and a release pattern that consisted of a high initial burst. Furthermore, structural stability of the released BSA was observed with separate PLGA microspheres but not with the PLGA/CPC. From both degradation and release data we can conclude that with PLGA/CPC, BSA readsorbed to the cement surface after being released from the (degraded)

microspheres. This process was mediated by the pH decrease that occurs as a result of PLGA microsphere degradation.

*Acknowlegdements*

Authors like to thank J.E. van den Dolder for helping with the SDS-PAGE experiments and H. op den Camp (Dept. of Microbiology, Radboud University Nijmegen, Nijmegen, The Netherlands) for the MALDI-TOF MS measurements. Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Center for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) R. Langer, J.P. Vacanti, Tissue engineering, Science. 260 (1993) 920-926

2) H. Seeherman, J.M. Wozney, Delivery of bone morphogenic proteins for orthopedic tissue regeneration, Cytokine Growth Factor Rev. 16 (2005) 329-345

3) J.A. Jansen, J.W.M. Vehof, P.Q. Ruhé, H. Kroeze-Deutman, Y. Kuboki, H. Takita, E.L. Hedberg, A.G. Mikos, Growth factor-loaded scaffolds for bone engineering, J Control Release. 101 (2005) 127-136

4) L. Comuzzi, E.M. Ooms, J.A. Jansen, Injectable calcium phosphate cement as a filler for bone defects around oral implants: an experimental study in goats, Clin Oral Implants Res. 13 (2002) 304-311

5) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/CaP cements: physical propetries and degradation characteristics, J. Biomater Sci Polym Ed. 17(9) (2006) 1057-1074

6) E.K Moioli, L. Hong, J. Guardado, P.A. Clark, J.J. Mao, Sustained release of TGDbeta3 from PLGA microspheres and its effect on early osteogenic differentiation of human mesenchymal stem cells, Tissue Eng. 12(3) (2006) 537-546

7) C. Carrascosa, L. Espejo, S. Torrado, J.J. Torrado, Effect of gamma-sterilization process on PLGA microspheres loaded with insuline-like growth factor-1 (IGF-1), J Biomater Appl. 18(2) (2003) 95-108

8) T.W. King, C.W. Patrick Jr., Development and in vitro characterization of vascular endothelial growth factor (VEGF)-loaded poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) microspheres using a solid encapsulation/single emulsion/solvent extraction technique, J Biomed Mater Res Part A 51(3) (2000) 383-390

9) C.H. Zheng, J.Q. Gao, W.Q. Liang, H.Y. Yu, Y.L. Zhang, Effects of additives and processing parameters on the initial burst release of protein from poly(lactic-co-glycolic acid) microspheres, PDA J Pharm Sci Technol. 60(1) (2006) 54-59

10) C. Bouissou, J.J. Rouse, R. Price, C.F. van der Walle, The influence of surfactant on PLGA microsphere glass transition and water sorption: remodeling the surface morphology to attenuate the burst release, Pharm Res. 23(6) (2006) 1295-1305

11) H.P. Stallmann, C. Faber, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, In vitro gentamycin release from commercially available calcium-phosphate bone substitutes influence of carrier type on duration of the release profile, BMC Musculosketal Disorders. 7(18) (2006) 1-8

12) E.J Blom, J. Klein-Nulend, J.G.C. Wolke, K. Kurashina, M.A.J. van Waas, E.H. Burger, Transforming growth factor-β1 incorporation in an α-tricalcium phosphate monoxide cement: release characteristics and physicochemical properties, Biomaterials. 23 (2002) 1261-1268

13) H.P. Stallmann, C. Faber, E.T. Slotema, D.M. Lyaruu, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11(hLF1-11) from calcium phosphate bone substitutes, J Antimicrob Chemother. 52 (2003) 853-855

14) J.K. Tessmar, A.M. Göpferich, Matrices and scaffolds for protein delivery in tissue engineering, Adv Drug Del Rev. 59(4-5) (2007) 274-291

15) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, RhBMP-2 release from injectable poly(DL-lactic-co-glycolic acid)/calcium phosphate cement composites, J Bone Joint Surg Am. 85A(suppl.3) (2003) 75-81

16) P.Q. Ruhé, O.C. Boerman, F.G.M. Russel, P.H.M. Spauwen, A.G. Mikos, J.A. Jansen, Controlled release of rhBMP-2 loaded poly(DL-lactic-*co*-glycolic acid)/Calcium phosphate cement composites in vivo, J Control Release. 106(1-2) (2005) 162-171

17) K. Schwartz, M. Epple, A detailed characterization of polyglycolide prepared by solid-state polycondensation reaction, Macromol Chem Phys. 200(10) (1999) 2221-2229

18) U. Hempel, A. Reinstorf, M. Pompe, U. Fisher, M. Gelinsky, W. Pompe, K.W. Wenzel, Proliferation and differentiation of osteoblasts on Biocement D modified with collagen type I and citric acid, J Biomed Mater Res Part B Appl Biomater. 71(1) (2004) 130-143

19) A.A. Ignatius, O. Betz, P. Augat, L.E. Claes, In vivo investigation on composites made of resorbable ceramics and poly(lactide) used as bone graft substitutes, J Biomed Mater Res Part A. 58(6) (2001) 701-709

20) W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen, Introduction of gelatin microspheres into an injectable calcium phosphate cement, J Biomed Mater Res Part A. *in press*

21) N. Nihant, C. Schugens, C. Grandfills, R. Jérôme, P. Teyssié, Polylactide microparticles prepared by double emulsion/evaporation technique. I. Effect of primary emulsion stability, Pharm Res. 11(10) (1994) 1479-1484

22) G. Schwach, N. Oudry, S. Delhomme, M. Luck, H. Lindner, R. Gurny, Biodegradable microparticles for sustained release of a new GnRH antagonist—part I: Screening commercial PLGA and formulation technologies, Eur J Pharm Biopharm. 56 (2003) 327-336

23) H.K. Kim, H.J. Chung, T.G. Park, Biodegradable polymeric microspheres with "open/closed" pores for sustained release of human growth hormone, J Control Release. 112 (2006) 167-174

24) I.S. Harding, H. Rashid, K.A. Hing, Surface charge and the effect of excess calcium ions on the hydroxyapatite surface, Biomaterials. 26 (2005) 6818-6828

25) K. Skartsila, N. Spanos, Surface charge of hydroxyapatite: potentiometric titrations coupled with solubility measurements, J Colloid Interface Sci. 308 (2007) 405-412

26) D.T. Hughes Wassell, R.C. Hall, G. Embery, Adsorption of bovine serum albumin onto hydroxyapatite, Biomaterials. 16 (1995) 697-702

27) J-H. Kim, A. Taluja, K. Knutson, Y.H. Bae, Stability of bovine serum albumin complexed with PEG-ply(L-histidine) diblock copolymer in PLGA microspheres, J Control Release. 109 (2005) 86-100

28) G. Zhu, S.R. Mallery, S.P. Schwendeman, Stabilization of proteins encapsulated in injectable poly(lactide-co-glycolide), Nature Biotechn. 18 (2000) 52-57

29) M.A. Ibrahim, A. Ismail, M.I. Fetouh, A. Göpferich, Stability of insulin during the erosion of poly(lactic acid) and poly(lactic-co-glycolic acid), J Control Release. 106(3) (2005) 241-252

30) F. Kang, J. Singh, Conformational stability of a model protein (bovine serum albumin) during primary emulsification process of PLGA microspheres synthesis, Int J Pharm. 260 (2003) 149-156

31) A.P. Serro, M. Bastos, J. Costa Pessoa, B. Saramago, Bovine serum albumin conformational changes upon adsorption on titania and on hydroxyapatite and their relation with biomineralization, J Biomed Mater Res Part A, 70 (2004) 420-427

32) J. Ziegler, U. Mayr-Wohlfart, S. Kessler, K.-P. Günther, Adsorption and release properties of growth factors from biodegradable implants, J Biomed Mater Res Part A. 59 (2002) 422-428

33) H. Hidaka, N. Hanyu, M. Sugano, K. Kawasaki, K. Yamanuchi, T. Katsuyama, Analysis of human serum lipoprotein lipid composition using MALDI-TOF mass spectrometry, Ann Clin Lab Sci. 37(3) (2007) 213-221

# CHAPTER 4

## INTRODUCTION OF GELATIN MICROSPHERES INTO AN INJECTABLE CALCIUM PHOSPHATE CEMENT

*W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen*

J Biomed Mater Res Part A., published online January 14, 2008

## 1. Introduction

Bone defects caused by trauma or tumor resection and age related loss of bone mass induce a high demand for bone filling materials. Most synthetic alloplasts are not suitable for these types of applications because their mechanical/physical or biological properties are not sufficient, so in most cases autogenous bone is still used[1-2]. On the other hand, calcium phosphate compounds appear to be an attractive alternative, as they are osteoconductive and can reach to compression strengths that are comparable to bone[3]. Especially calcium phosphate cement (CPC) is a good candidate since it can be shaped to the defect site after injection and renders an optimal contact between bone and material[4]. Despite this, the *in vivo* resorption and tissue ingrowth of most CPCs is slow[5]. In previous studies[6-8] poly(*DL*-lactic-*co*-glycolic acid) (PLGA) microspheres were added to improve the properties of a CPC for tissue engineering purposes. These microspheres introduced macroporosity into the cement and were also applied for the delivery of growth factors. *In vitro* degradation tests[6] showed a slow degradation of the PLGA microspheres that was accompanied by a pH decrease of the surrounding medium. Compressive strength of the microsphere composites decreased concomitantly as the PLGA eroded, rendering highly macroporous scaffolds.

Next to PLGA also other polymers can be applied for the production of microspheres. Especially natural polymers like chitosan[9], collagen[10-11] and gelatin are interesting candidates that demonstrate enzymatic degradation. Of these polymers, gelatin would be the preferred material because it does not express antigenicity in physiological conditions and is completely resorbable *in vivo*, unlike collagen[12]. Gelatin microspheres have been used by different authors for the sustained release of growth factors[13-14]. Here, the loading of growth factor relies on an ion-binding interaction between an acidic or basic gelatin and a positively/negatively charged protein[15]. To stabilize gelatin microspheres in an aqueous environment a crosslinker[16-18] is used. Degradation characteristics of the microspheres can be tailored by changing the crosslinking density. Gelatin can be derived from different animal sources, though porcine (type A, basic) and bovine (type B, acidic) gelatin cover 95% of both household and industrial use. Food grade (FG) gelatin consists mostly of type A gelatin (80%) and less of type B (15%).

The goal of this study was to characterize the gelatin microsphere CPC on mechanical/ physical properties and degradation characteristics. For this purpose, food-grade (FG) gelatin microspheres with different crosslinking densities were incorporated into the cement. Injectability and setting/cohesive properties of the composites were determined. Furthermore, a 12-week *in vitro* degradation test in proteolytic medium was performed using preset scaffolds. Samples were assayed on mass loss, compressive strength, E-modulus and morphology. In addition to the *in vitro* degradation test, a degradation study with gelatin microspheres was performed to investigate the influence of physical conditions inside the cement like pH and calcium concentration on microsphere stability. Also degradation characteristics of type A and type B gelatin microspheres were compared to the food-grade gelatin.

## 2. Materials and Methods

### *2.1. Materials*

Gelatin (food-grade (FG), Granules, pH = 3.8-7.6, bloom number = 80-120, Merck, Darmstadt, Germany; type A (pI = 7.0-9.0, bloom number = 300) / type B (pI = 4.7-5.2, bloom number = 225), cell culture tested, Sigma-Aldrich, St. Louis, MO, USA) was used for the preparation of the microspheres. Olive oil (Acros Organics, Geel, Belgium) and acetone (HPLC grade, Labscan ltd., Dublin, Ireland) were used as substrate during microsphere preparation. Glutaraldehyde (25wt% solution, EM-grade, Merck, Darmstadt, Germany) was applied as crosslinker. The calcium phosphate cement (Calcibon®, Biomet Merck, Darmstadt, Germany) was composed of 61% α-TCP, 26% $CaHPO_4$, 10% $CaCO_3$ and 3% precipitated HA. The cement liquid applied was a 1% aqueous solution of $Na_2HPO_4$.

### *2.2. Methods*

#### *2.2.1. Preparation gelatin microspheres*

2.5 g of gelatin was dissolved in 25 ml $ddH_2O$ for 30 min at 60°C. While stirring at 500 rpm using a Teflon upper stirrer, the resulting clear solution was added slowly (10 ml pipette) to a 250 ml three-necked round bottom flask containing 125 ml olive oil. During stirring, the round bottom flask was put in an ice bath. After 30 min, 50 ml of chilled acetone (4°C) and glutaraldehyde (0.5 ml = 6.25 mM) was added slowly. The solution was stirred for another 1h (GEL1) or 2h (GEL2) to produce microspheres with a higher extent of crosslinking. Microspheres were collected by filtration (D3, Schott Duran, Mainz, Germany) and washed several times with acetone (approx. 1 l) to remove residual olive oil. Following this, microspheres were dried over night in a vacuum stove. For the enhanced degradation test all microspheres were crosslinked for 1h.

#### *2.2.2. Preparation of gelatin microsphere CPC*

Gelatin microspheres were swollen before cement powder or liquid hardener was added to obtain a good distribution of microspheres inside the cement. Therefore, 50 mg of dried GEL1/GEL2 microspheres were put in a 2 ml plastic syringe, subsequently adding 300 µl/250 µl demineralized water ($ddH_2O$) after which the syringe was stirred vigorously for 15 s using a mixing device (Silamat® mixing apparatus, Vivadent, Schaan, Liechtenstein). Subsequently, 950 mg cement powder was added to the swollen microspheres and the resulting mixture was stirred for another 15 s. Then 300 µl of hardener solution (1% $Na_2HPO_4$) was added and the content was stirred again for 15 s. The resulting cement paste was injected into a Teflon mould (6 cylinders, 4.5 x 10 mm), after which samples were left to set at room temperature for 24 hr and stored in a vacuum stove.

*2.2.3. Morphology analysis*

The morphology of the gelatin microspheres and gelatin microsphere CPC was evaluated by scanning electron microscopy (SEM) (JEOL 6400-LINK AN 10000 at 10 kV). The samples were mounted on aluminum stubs using carbon tape and sputter-coated with gold-palladium prior to examination.

*2.2.4. Size distribution*

The particle size distribution of the gelatin microspheres was determined by image analysis. Dry microspheres were visualized by SEM, while swollen microspheres were characterized with an optical microscope (Leica) after suspension in $ddH_2O$. The size distribution of both dry and swollen spheres was determined using digital image software (Leica Qwin).

*2.2.5. Injectability*

After mixing, the syringe, with an orifice diameter of 1.7 mm, was fitted vertically in a fixture and put under the platen of a tensile bench set (858 MiniBionixII®, MTS Corp., Eden Prairie, MN, USA) in tension mode[6]. The cement composites were compressed until approximately 1 cc was present inside the syringes, which was used as starting point. 25 seconds after mixing the cement (for 15s), a compressive force was applied to the syringe with a constant velocity of 10 mm/min. The compressive force was recorded as a function of the plunger travel length[19] after which it was converted into time. The time from mixing the paste until pressure reaches 60N, is referred to as the injection time. If all the paste is injected before pressure increase, the paste is regarded as fully injectable. All tests were performed threefold (n = 3).

*2.2.6. Setting time*

The initial and final setting times were assessed using custom available Gillmore needles (ASTM C266). A bronze block was used as mould containing 6 holes (6 mm in diameter, 12 mm in height). The mould was placed in a water bath to simulate body temperature (37˚C). Samples were mixed and injected into the mould in a retrograde fashion, after which the initial and final setting time of the samples was determined (n = 3).

For determination of the cohesive properties of the gelatin microsphere CPC, samples were injected into Ringer's solution at 37°C. During the cement setting time it was observed whether the paste retained its original configuration or powder formation/disintegration occurred. Every anomaly was recorded.

*2.2.7. Porosity*

The macro- and total porosity of preset gelatin microsphere CPC samples was determined. The macroporosity is the porosity in which the pores are created by the degradation of gelatin

microspheres. The total porosity is the macroporosity plus the original microporosity of the cement. When the gelatin microspheres are not degraded, the macroporosity corresponds to the vol% of microspheres initially present inside the cement.

To measure these parameters, both gelatin microsphere CPC samples and microporous CPC samples of a known volume were placed in an oven at 650°C for 2 h. After burning out the gelatin/moisture, samples were weighed and Equation 1 and 2 were used for the derivation of the total porosity and the macroporosity. Tests were performed threefold (n = 3).

$$\varepsilon_{tot} = (1 - \frac{m_{macro/micro}}{V * \rho_{HAP}}) * 100\% \qquad \textit{Equation 1}$$

$$\varepsilon_{macro} = (1 - \frac{m_{macro}}{m_{micro}}) * 100\% \qquad \textit{Equation 2}$$

*Legend:* $\varepsilon_{tot}$ = total porosity (%), $\varepsilon_{macro}$ = macroporosity (%), $m_{macro}$ = average mass macroporous sample (after burning out gelatin) (g, n=3), $m_{micro}$ = average mass microporous sample (g, n=3), V = volume sample ($cm^3$), $\rho_{HAP}$ = density hydroxy apatite ($g/cm^3$)

*2.2.8. Degradation assay*

For the degradation assay gelatin microsphere CPC samples were prepared as described before. Samples were placed in 3 ml of phosphate-buffered saline (PBS, pH 7.4) containing 50 μg/ml gentamycin and 373 ng/ml collagenase 1A(Sigma)[13] and incubated at 37˚C in a water bath on a shaker table (70 rpm) for 12 weeks. Every 3-4 days sample buffer was refreshed. At days 1, 3, 7, 14, 28, 42 and 84, specimens were subjected to analysis according to the descriptions summarized below. Samples were taken fivefold (n = 5). Gelatin microspheres (8-9 mg, dry spheres) and microporous cement scaffolds were used as a control.

pH measurements

After removal of the samples from the water bath the pH of the PBS medium was measured.

Mass loss quantification

Samples were freeze-dried overnight before measuring the mass. The mass loss of the samples was calculated using Equation 3.

$$R_L = \frac{M_0 - M_n}{M_0} * 100\% \qquad \textit{Equation 3}$$

*Legend:* $R_L$ = mass loss sample at t = n (%), $M_0$ = mass sample at t = 0 (g), $M_n$ = mass sample at t = n (g)

Morphology

The morphology of the samples was determined using SEM. To visualize composite degradation and increase in (macro)porosity, overview pictures were taken at a magnification of 100x. Also, pictures were taken at higher magnifications to visualize the microsphere degradation. Next to that, energy dispersive X-Ray spectrometry (EDS) was performed for further material analysis.

Mechanical characteristics

Samples were placed in a mechanical testing bench (858 MiniBionixII®, MTS Corp., Eden Prairie, MN, USA) and both compressive strength and E-modulus along the height of the specimens were measured at a crosshead speed of 0.5 mm/min.

*2.2.9. Supplementary microsphere degradation study*

In addition to the 12 weeks degradation study, a degradation study using gelatin microspheres was performed to investigate the influence of physical conditions inside the cement like pH and calcium concentration on microsphere stability. Also the degradation characteristics of FG gelatin microspheres were compared to microspheres of type A and type B gelatin. In this study the degradation of the microspheres was enhanced by applying a high concentration of gelatin-degrading enzyme, which enabled us to compare the degradation characteristics of these microspheres within a short time period. For this purpose 10 mg of 1h crosslinked gelatin microspheres of either type A, B or FG were put in 3 ml PBS containing 10μg/ml collagenase 1A at 37°C. Applying these conditions, a pilot study showed that FG microspheres were fully degraded within 3 days. Because an exponential degradation profile was observed in this pilot, a time period of 1 day was chosen for the degradation study. To mimic the conditions inside the gelatin microsphere CPCs, gelatin microspheres were pretreated for 1 day in PBS containing 0.1M $CaCl_2$ (pH = 5.72) before they were exposed to the enhanced degradation medium. PBS (pH = 7.4) and acidic PBS (pH = 5.72) were taken as a control. Samples were assayed on mass loss and morphology (n = 3).

*2.2.10. Statistical analysis*

Data were arranged as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat®.

## 3. Results

### *3.1. Preparation microspheres*

SEM-micrographs of the prepared microspheres are shown in Figure 1A-B. Dry microspheres were partially agglomerated and imprints of the interconnections between the spheres were visible at the surface. In an aqueous environment, the GEL1 and GEL2 microspheres showed an approximately 1.5 times increase in diameter (Table 1) during which the interconnections were broken. The size distribution (Figure 1C) showed that most unswollen microspheres were below 10 μm in diameter and an increase in sphere size higher than 30μm was observed after swelling. Overall, a slightly higher percentage of larger spheres was observed in the GEL1 group.

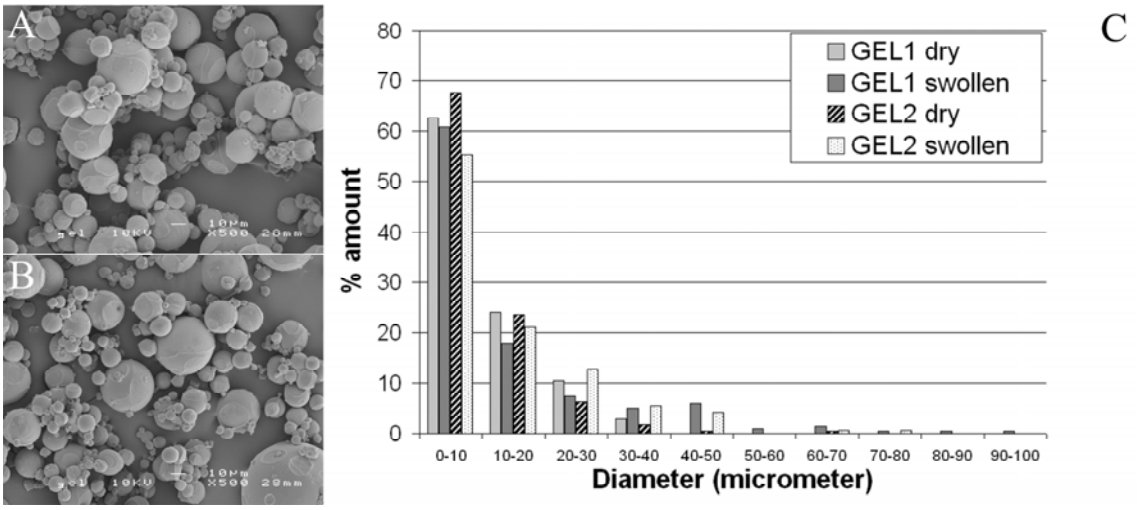



**Figure 1.** SEM image of the morphology of GEL1 microspheres (A), GEL2 microspheres (B) and size distribution of dry and swollen microspheres (C), group size: n=171/166 (dry microspheres), n=201/222 (swollen microspheres)

**Table 1.** Physical parameters cement/microsphere formulations

| | Microporous[6] | GEL1 CPC | GEL2 CPC |
|---|---|---|---|
| Average microsphere size (μm) | - | 9.70 (dry)<br>14.79 (swollen) | 8.64 (dry)<br>13.76 (swollen) |
| Initial setting time (s) | 108.3 ± 5.8 | 165.0 ± 15.0 | 145.0 ± 8.7 |
| Injection time (s) | 108 | 128 | 120 |
| Mass (mg) | 262.7 ± 6.1 | 166.0 ± 5.3 | 174.2 ± 5.5 |
| Porosity * | 45.52± 1.26 | 69.67 ± 1.29 | 69.21 ± 0.36 |
| Macro porosity * | - | 44.32 | 43.49 |

* = after removing gelatin microspheres

### *3.2. Preparation & clinical handling properties gelatin microsphere CPC*

A SEM-micrograph of the microspheres inside the cement is given in Figure 2A, which is representative for both GEL1 and GEL2 microsphere CPCs. A substantial percentage of the spheres was broken, showing an eggshell structure. Furthermore, an equal distribution of microspheres inside the cement was obtained. The injectability graph of the gelatin microsphere CPCs (Figure 2B) showed that the pressure on the syringe increased at a later time point than the microporous cement[6], indicating that the composite cement was easier to inject. When comparing both gelatin microsphere CPCs, the GEL1 composite showed a longer injection time, i.e. better injectability than the GEL2 composite. The setting time (Table 1) of both gelatin microsphere CPCs was comparable to each other and slightly higher than the microporous cement ($p<0.01$). No final setting time was measured due to the high water content of the gelatin microspheres. Cohesion tests revealed no powder formation or disintegration when injecting the samples in an aqueous environment. The macroporosity of both GEL1 and GEL2 microsphere CPCs was 43-44%, with a total porosity of 69-70%.

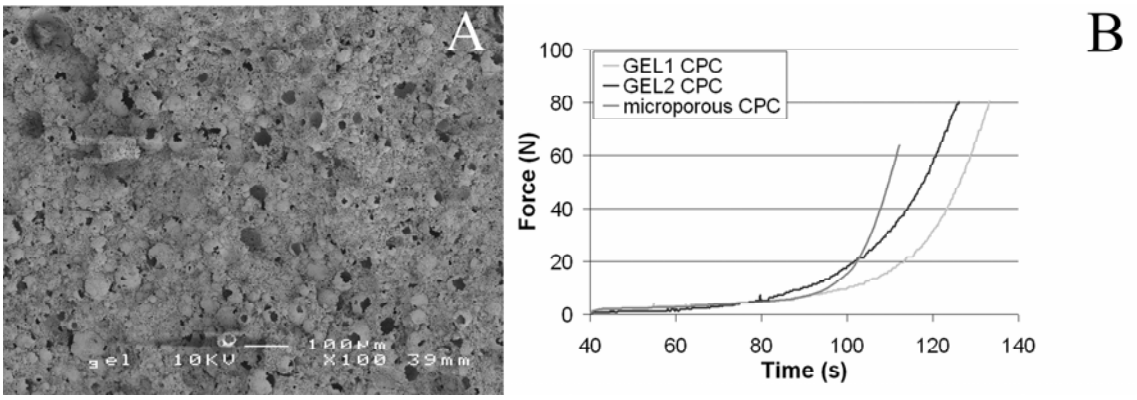



**Figure 2.** Morphology gelatin microsphere CPC (A) and injectability graph of GEL1/GEL2 microsphere CPC and microporous CPC[6] (B)

### *3.3. Degradation assay*

In Figure 3A the cumulative pH of the PBS medium is given as a function of the degradation time. The medium of both gelatin microsphere CPCs showed an initial pH increase, followed by a decrease and stabilized at a pH of 6.1 at t = 40 days. The release medium of both GEL1 and GEL2 microspheres showed a small decrease to a pH of 7.1. The development of the mass in time is shown in Figure 3B. For both gelatin microsphere CPCs an initial increase of 2% was followed by a slow decrease in mass. After 12 weeks a maximum decrease of 2% was observed. The microporous cement only showed an increase in mass of 3% that appeared to decrease after 12 weeks. Significant differences in mass loss between the microporous cement and gelatin microsphere CPCs were observed ($p<0.05$ at t = 4, 8 weeks), though only the GEL1 microsphere CPC showed a significant decrease in mass at t = 12 weeks ($p<0.01$). The gelatin microspheres showed an initial increase in mass that was higher than the increase of the gelatin microsphere CPCs, but decreased very rapidly after 3 days. Significant differences between the GEL1 and GEL2 microspheres were observed at day 1 and 14 ($p<0.05$), although the trend was similar.

The development of the compression strength of the gelatin microsphere CPCs over time is given in Figure 4A. An initial increase was observed over 7 days, after which the compression strength remained almost constant for 12 weeks. The final compression strength of both gelatin microsphere CPCs was 30 MPa, which was significantly lower than the microporous cement ($p<0.001$). The E-modulus (Figure 4B) showed a comparable trend with respect to the compression strength with an increase till day 7 after which it remained stable at ± 2000 MPa. Also here the microporous cement was significantly higher ($p<0.05$) and no significant differences were observed between the GEL1 and GEL2 composites.

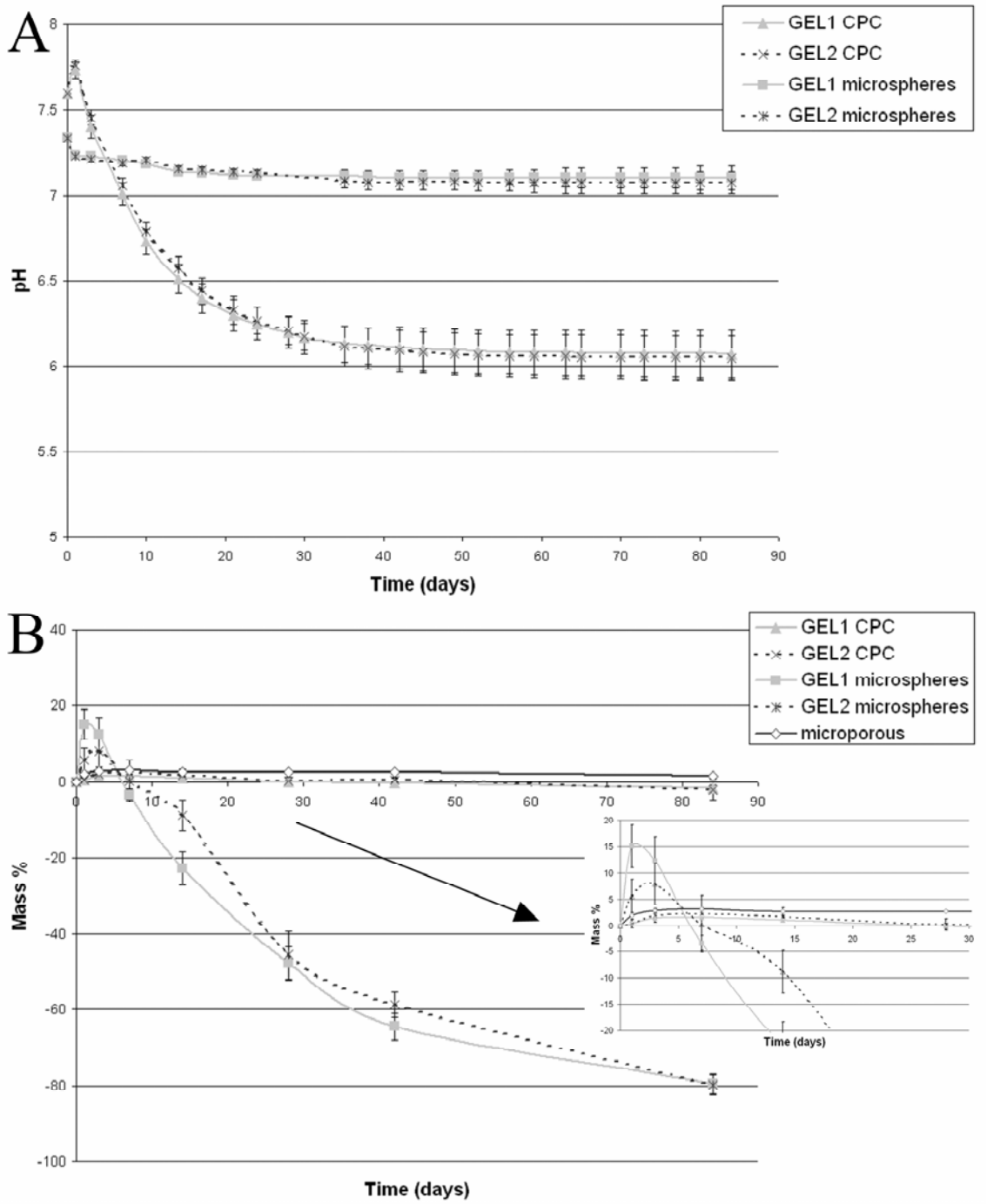



**Figure 3.** pH decrease of surrounding medium (A) and mass loss (B) as a function of degradation time of the GEL1/GEL2 microsphere CPC, microspheres and microporous cement

The morphology of the gelatin microsphere CPCs and gelatin microspheres separately are given in Figure 5-6. For both gelatin microsphere CPCs small differences in morphology were observed between samples from day 1 to week 12. Microsphere structures were still visible after 12 weeks and no increase in macroporosity was observed during the degradation test. In contrast, comparison of samples taken at day 1 with samples taken at later time points showed even a clear densification of the material. At higher magnifications (Figure 7), the presence of crystals onto/inside the gelatin

microspheres was observed. EDS analysis revealed that these crystals consisted of calcium and phosphate which indicated a recristallization of the cement at these sites.

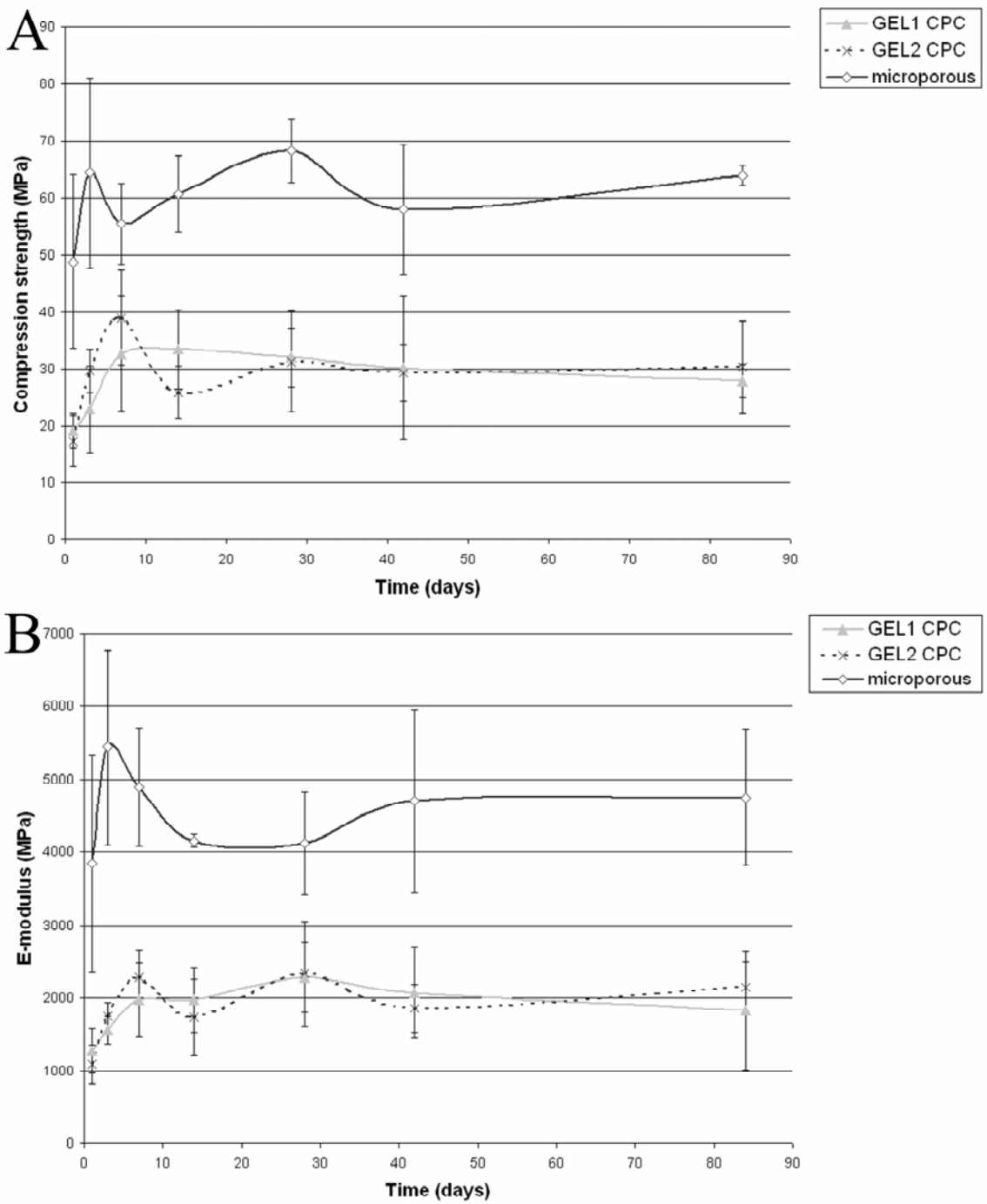



**Figure 4.** Compression strength (A) and E-modulus (B) as a function of degradation time of the GEL1/GEL2 microsphere CPC and microporous cement

The separate gelatin microspheres showed already deformations at day 1, slowly losing their spherical shape and fusing into a network of gelatin particles at week 4 and 6. After 12 weeks no separate gelatin microspheres were distinguished anymore. The structures still visible with SEM were traced to accumulations of PBS salts and some salts remaining from the gelatin. Differences between the GEL1

and GEL 2 microspheres were visible especially at 4 weeks where the remaining structures of the GEL2 microspheres showed a smaller, more particle-like morphology.

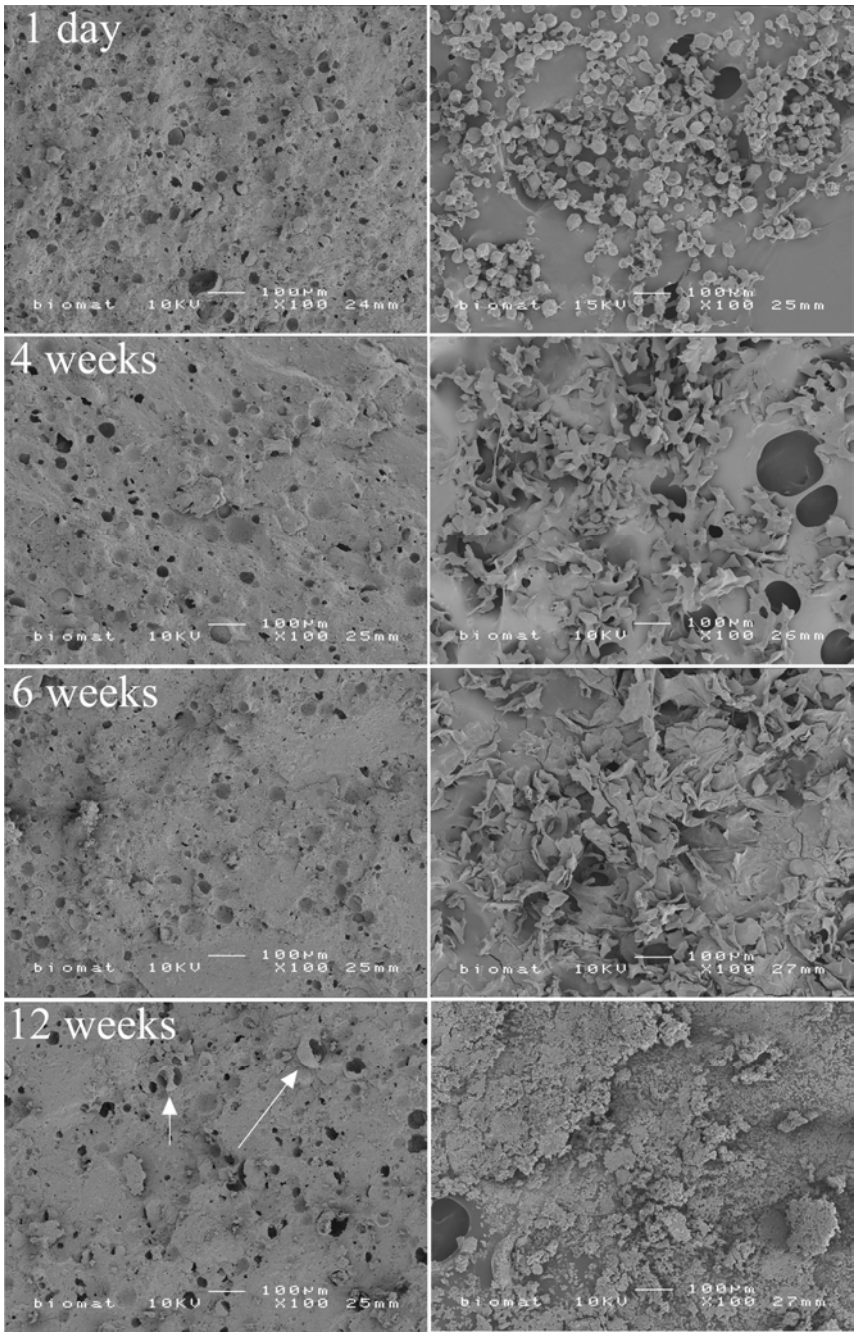



**Figure 5.** SEM-micrographs of GEL1 microsphere CPC(left) and GEL1 microspheres (right) at t =1 day and 4,6 and 12 weeks (original magnification 100x), arrows indicate the presence of gelatin microsphere shells

### *3.4. Supplementary microsphere degradation study*

Figure 8 shows the % mass left after 1 day in degradation medium using three types of gelatin and three different pretreatment methods. The results showed that the pretreatment method was important since both PBS + $CaCl_2$ and PBS (pH = 5.72) groups degraded significantly slower than the PBS (pH = 7.4) group with all types of gelatin (Table 2).

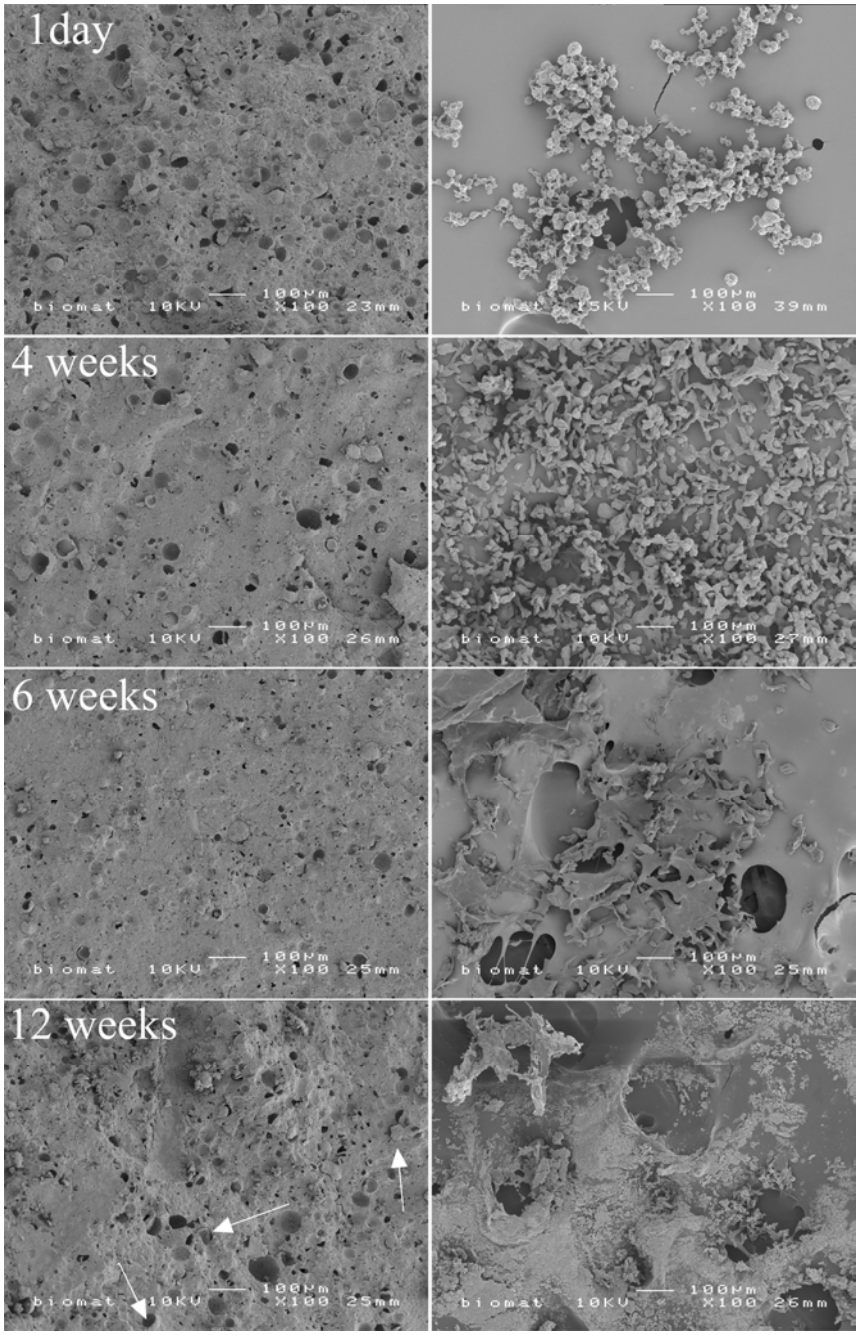



**Figure 6.** SEM-micrographs of GEL2 microsphere CPC(left) and GEL2 microspheres (right) at t =1 day and 4,6 and 12 weeks (original magnification 100x), arrows indicate the presence of gelatin microsphere shells

A difference between the PBS (pH = 5.72) group and the PBS + $CaCl_2$ group was observed with type B gelatin that exhibited a higher mass loss with the PBS + $CaCl_2$ group. Furthermore it was observed that both type A and type B gelatin degraded significantly faster than the FG gelatin within all pretreatment groups.

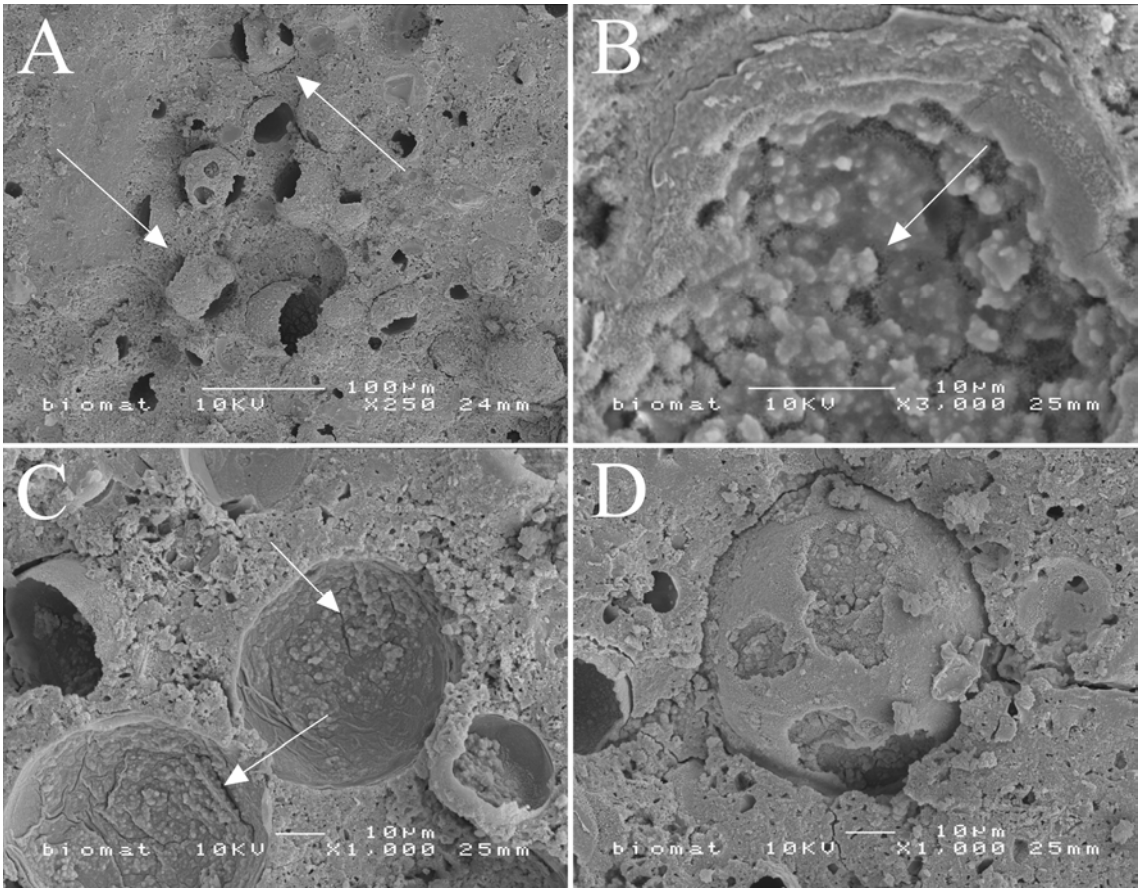



**Figure 7.** Close-up pictures of gelatin microspheres inside the cement, showing precipitate onto/inside the gelatin microspheres (arrows); A = GEL1 3 days (original magnification 250x), B = GEL1 2 weeks (original magnification 3000x), C = GEL1 6 weeks (original magnification 1000x), D = GEL2 6 weeks (original magnification 1000x)

Figures 9-11 depict SEM-micrographs of gelatin type A, type B and FG microspheres before and after pretreatment and after 1 day in enhanced degradation medium. Before pretreatment only perfect spheres were present, after pretreatment the spheres were deformed within all groups. After 1 day in enhanced degradation medium, microparticle structure was lost in the PBS (pH=7.4) groups, while with the PBS+$CaCl_2$ and PBS (pH=5.72) groups microparticles or a structure of elongated/connected microparticle structures were still visible. Also, morphological degradation was found to differ between the various types of gelatin. Microspheres prepared of FG gelatin maintained their microsphere structure very well, while type A gelatin microspheres showed deformations into an agglomerate structure within all pretreatment groups. Type B gelatin was intermediate, with the PBS+$CaCl_2$-group showing a separate microparticle structure and the PBS (pH=5.72) group showing an agglomerate structure consisting of thin, elongated microparticles.

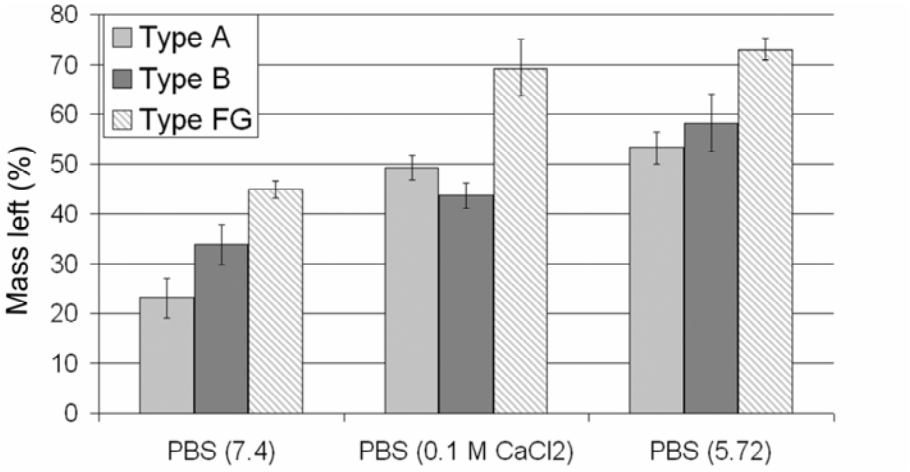



**Figure 8.** Mass remaining at t=1 day of enhanced degradation with Type A, Type B and FG gelatin microspheres after pre-treatment in PBS with pH=7.4, 0.1M $CaCl_2$ or pH=5.72

**Table 2.** Statistical significance samples supplementary degradation study

| Parameter | Samples | Significance |
|---|---|---|
| Pretreatment method | type A PBS (7.4) vs type A PBS+$CaCl_2$ | $p < 0.001$ |
| | type A PBS (7.4) vs type A PBS (5.72) | $p < 0.001$ |
| | type B PBS (7.4) vs type B PBS+ $CaCl_2$ | $p < 0.05$ |
| | type B PBS (7.4) vs type B PBS (5.72) | $p < 0.001$ |
| | type B PBS+ $CaCl_2$ vs type B PBS (5.72) | $p < 0.001$ |
| | type FG PBS (7.4) vs type FG PBS + $CaCl_2$ | $p < 0.001$ |
| | type FG PBS (7.4) vs type FG PBS (5.72) | $p < 0.001$ |
| Type of gelatin | type A PBS (7.4) vs type B PBS (7.4) | $p < 0.05$ |
| | type A PBS (7.4) vs type FG PBS (7.4) | $p < 0.001$ |
| | type B PBS (7.4) vs type FG PBS (7.4) | $p < 0.01$ |
| | type A PBS + $CaCl_2$ vs type FG PBS+ $CaCl_2$ | $p < 0.001$ |
| | type B PBS+ $CaCl_2$ vs type FG PBS+ $CaCl_2$ | $p < 0.001$ |
| | type A PBS (5.72) vs type FG PBS (5.72) | $p < 0.001$ |
| | type B PBS (5.72) vs type FG PBS (5.72) | $p < 0.001$ |

## 4. Discussion

In this study gelatin microspheres were added to a microporous calcium phosphate cement to investigate the possibility of these composite materials to serve as an injectable bone substitute, and secondly as a possible creator of macroporosity after gelatin degradation. For this purpose, food grade (FG) gelatin microspheres with different crosslinking density were added to the cement and subjected to physical/chemical characterization. An *in vitro* degradation study with these composites was performed in proteolytic degradation medium to see whether macroporosity could be created. Furthermore, to investigate the influence of physical conditions inside the cement like pH and calcium concentration on microsphere stability, a supplementary degradation study was performed. In this degradation study also microspheres of cell-culture-tested-gelatin type A and type B were investigated and compared to the FG gelatin microspheres.

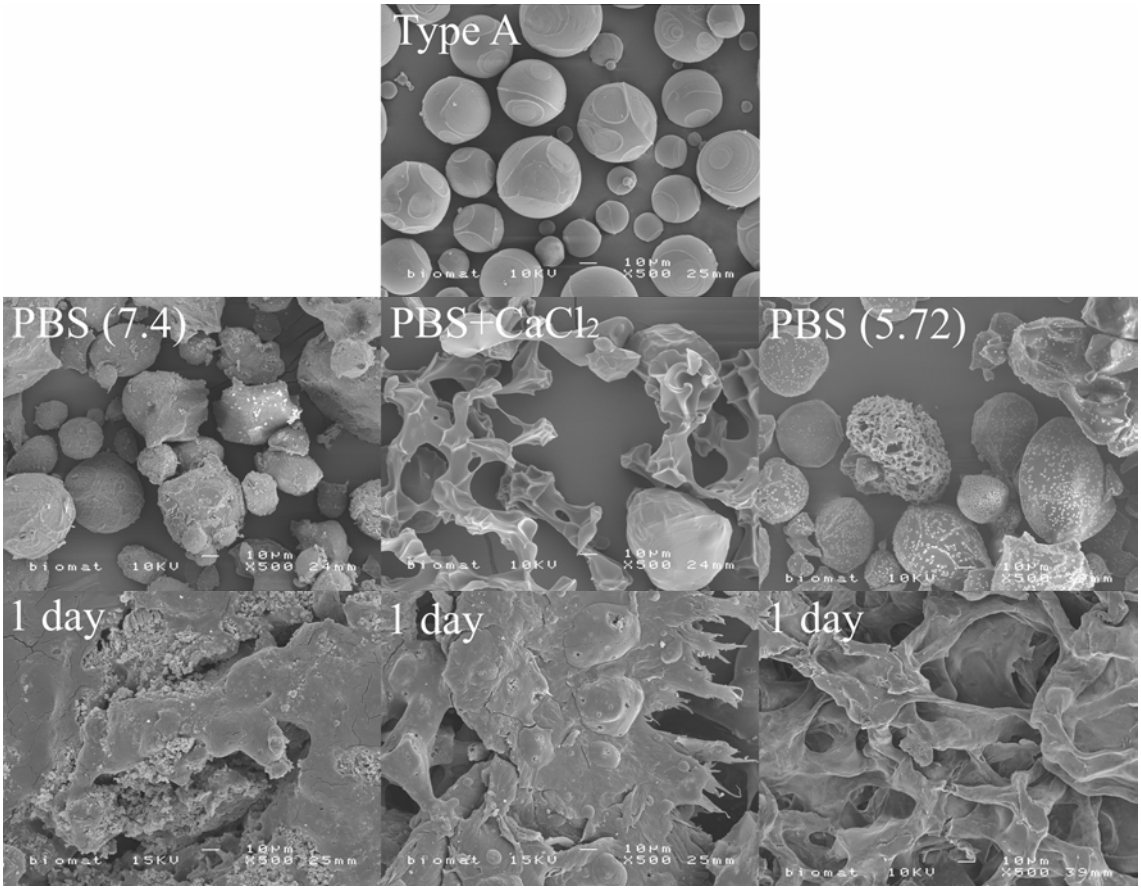



**Figure 9.** SEM-micrographs of Type A gelatin microspheres before pretreatment (upper), after pretreatment in PBS with pH= 7.4, 0.1M $CaCl_2$ or pH=5.72 (middle) and after t=1 day of enhanced degradation study (lower) (original magnification 500x)

The physical characteristics of the cement with added gelatin microspheres differed significantly from the microporous cement. Initial setting time showed a significant increase and final setting time was not measurable. An explanation for this is that the gelatin microspheres were added to the cement in a swollen state to obtain a good distribution. The water inside these microspheres contributed to cement setting, leading to increased setting times[20]. On the other hand, the addition of gelatin microspheres to the cement resulted in improved injection properties, as injection time increased for the gelatin microsphere CPCs when compared to microporous cement. Differences in injectability were observed between the GEL1 and GEL2 composite where increased crosslinking of the gelatin microspheres led to more stiffer structures[21]. After cement setting, SEM revealed that the microspheres inside the gelatin microsphere CPCs showed a structure of broken, hollow spheres. It can be hypothesized that physical bonds between the gelatin microspheres and surrounding cement were responsible for this structure as shrink stresses, arisen during drying, forced the immobilized spheres to break.

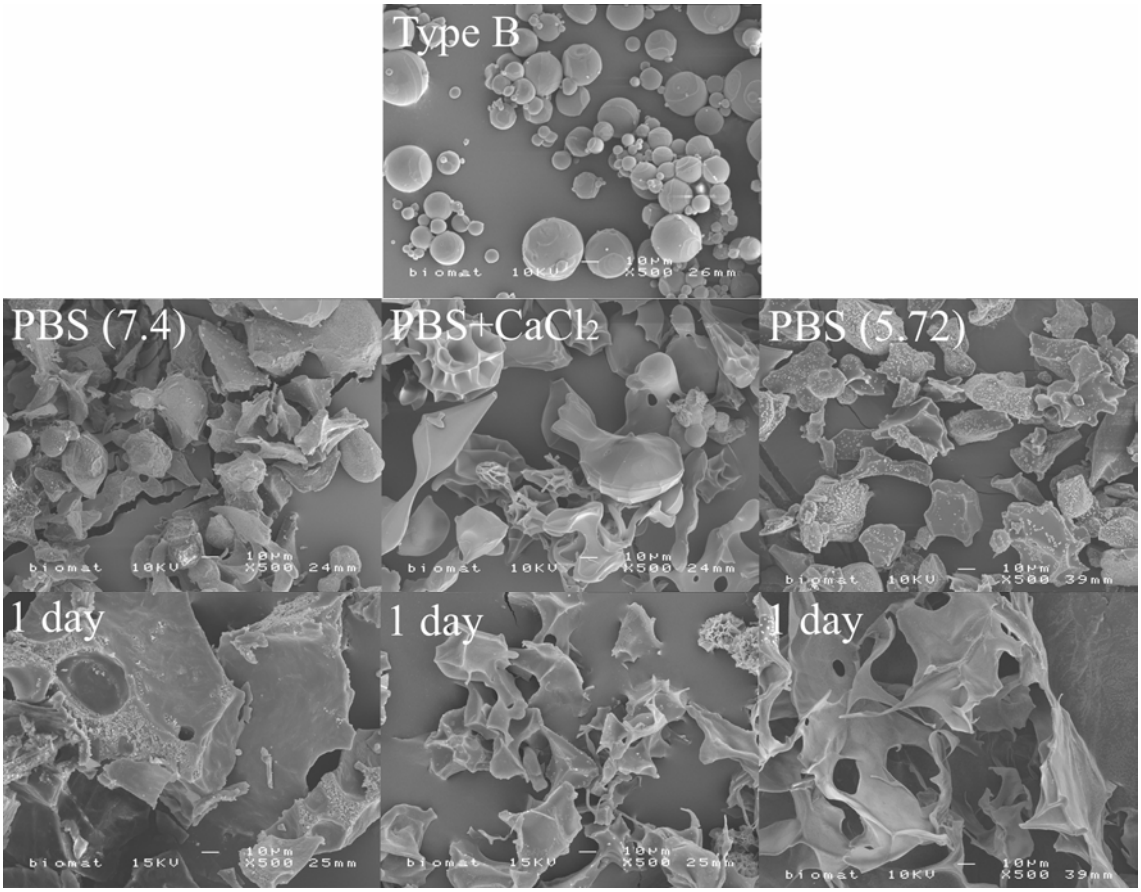



**Figure 10.** SEM-micrographs of Type B gelatin microspheres before pretreatment (upper), after pretreatment in PBS with pH= 7.4, 0.1M $CaCl_2$ or pH=5.72 (middle) and after t=1 day of enhanced degradation study (lower) (original magnification 500x)

The degradation experiment showed an initial increase in mass for the gelatin microsphere CPCs at day 1 and 3. This increase in mass was also observed in a higher extent with the separate gelatin microspheres and the microporous cement. An explanation is the precipitation of PBS crystals on the outside of the cement samples and microspheres during the experiment. The high increase observed with separate gelatin microspheres was caused by a similar mechanism. Here, weight was measured after gently removing the medium above the microsphere gel and freeze-drying the microspheres. Because the swollen gelatin microspheres contained about 6 times their wt% of water, they also retained a PBS precipitate that contributed to the weight of the samples. Rinsing the microspheres with demineralized water before drying is not a solution, since this will greatly influence the mass of the microspheres themselves as degradation products dissolve during rinsing and microspheres will get lost.

The initial decrease in pH was also observed in a previous experiment[6] and is due to the release of acidic products from the cement, while the microspheres themselves only show a minimal decrease in pH.

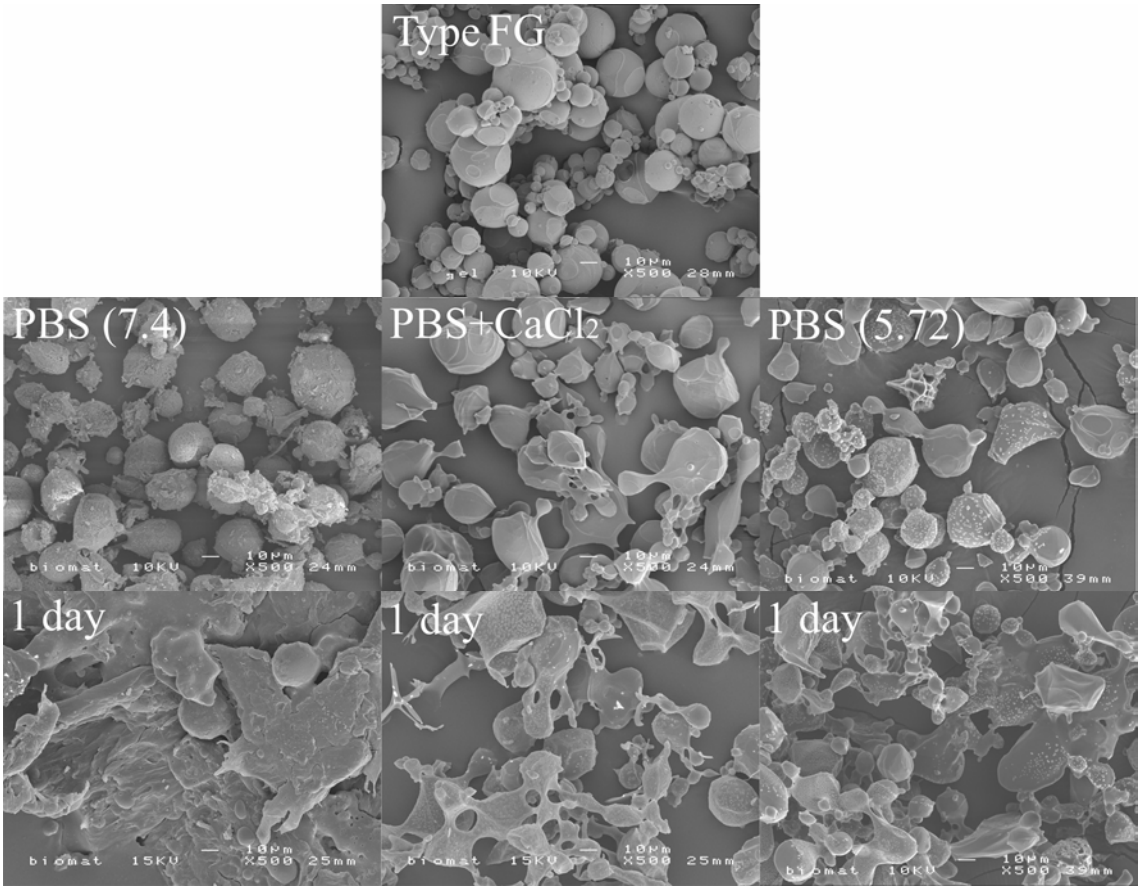



**Figure 11.** SEM-micrographs of Type FG gelatin microspheres before pretreatment (upper), after pretreatment in PBS with pH= 7.4, 0.1M $CaCl_2$ or pH=5.72 (middle) and after t=1 day of enhanced degradation study (lower) (original magnification 500x)

The gelatin microsphere CPCs also showed a striking increase in mechanical strength within the first 7 days. This increase is due to the recristallization of the cement into hydroxyapatite, which is well-described in literature[20]. In addition, the clear densification of the cement observed with SEM is most likely also caused by cement recristallization. Because of the biomimetic properties of the gelatin[12], the fractured microspheres showed a shell of calcium phosphate (CaP) precipitate onto the surface forming a strong bond between the cement and the gelatin. Comparison of the current results with PLGA microsphere CPCs in a previous experiment[6], the PLGA microspheres were always present without a shell of CaP precipitate. At later time points (>2 weeks) even the formation of crystals inside the gelatin microspheres was observed, indicating that the pores formed by the microspheres can be filled with a precipitate.

At the end of the degradation test, the obtained scaffold did not show an increase in macroporosity or increased cement resorption. A first explanation for these results is the occurrence of the discussed CaP precipitates onto and inside gelatin microspheres. The volume % of gelatin microspheres inside the composite should be sufficient to form interconnections between the microspheres. However, if CaP is precipitating between two contacting microspheres, no interconnection will be formed but a shell of crystalline hydroxyapatite (HA). Increasing the % of microspheres inside the cement, can solve this problem partially as the influence of the cement will be smaller and more interconnections

will be present. On the other hand, precipitation will still occur and the addition of more than 5wt% of microspheres greatly increases setting time and decreases mechanical properties[6,20].

Secondly, the weight loss of the GEL1 and GEL2 microsphere CPCs was very small when compared to the microporous cement and did not exceed the 5wt% of microspheres added initially. If the gelatin microspheres inside the cement composite had degraded as fast as the separate gelatin microspheres, the composites should have shown a higher decrease in mass and distinct morphological degradation of the microspheres after 6-12 weeks. SEM-pictures at these time points still showed the presence of intact gelatin shells throughout the composite and therefore it can be concluded that the microspheres degraded slower inside these composites. An explanation for the delayed degradation is binding/deactivation of the enzyme to the CPC. Since gelatin degrades by an enzymatic route, the calcium phosphate matrix of the gelatin microsphere CPC can prevent the enzyme from entering the material. Collagenase is prone to bind to CPC, which was proven to have a protein-binding capacity[7,22-23]. According to Kremer et al.[24] a suspension of hydroxyapatite crystals in combination with free $Ca^{2+}$-ions (>0.5 mM) even can induce an autolytic degradation of matrix metalloprotease-1 (MMP-1, interstitial collagenase). A decrease in pH, as observed with the gelatin microsphere CPCs, also could have influenced collagenase activity. However, the optimal pH range for collagenase is 6.3-8[25] and individual pH values measured during the test never dropped below 6.8.

From the supplementary microsphere degradation test it can be concluded that a small pH decrease as is present inside the microporous cement[6], delays gelatin microsphere degradation by changing the microsphere structure/stability. This is most likely caused by the pH-dependent crosslink reaction of residual aldehyde groups after subjecting the microspheres/composite to the degradation medium. In this experiment gelatin microspheres were crosslinked with glutaraldehyde to ensure structural stability of the spheres during cement mixing. The amount of glutaraldehyde used (6.25 mM) and crosslink time (1h minimum) were just sufficient for sphere stability because a fast degrading microsphere was proposed to give a superior cement composite. However, despite this low amount of crosslinker, with the applied crosslink method free aldehyde groups were still present after microsphere preparation as glutaraldehyde partially exists as oligomers with multiple aldehyde chains[26]. In aqueous conditions, for example the degradation medium, these aldehyde chains can further react with free amino groups, preferentially lysine or arginine in the gelatin[27] which leads to the formation of new crosslinks, in particularly Schiff bases ($R_1R_2C=N-R_3$, with $R_3$= alkylic or arylic)[28-29]. As aldehyde groups become more electrophilic at a lower pH[30], the modest pH decrease must have accelerated these crosslink reactions thereby stabilizing the microspheres against proteolytic degradation. An approach to prevent this delayed crosslink reaction is for example the addition of an aldehyde reagent (inhibitor) to the oil phase at the end of microsphere preparation. Glycin[13,31] is commonly used as it is a body-own substance. Also, the combined used of citric acid and citric acic/glycin[32] shows inhibition of the crosslink reaction.

Next to covalent bonding of gelatin chains, non-covalent bonding also played a small role in the gelatin microsphere CPC degradation as was observed in the supplementary degradation test. $Ca^{2+}$-ions are present inside the degradation medium due to the dissolution of monetite and calcium carbonate. These $Ca^{2+}$ ions can bind to multiple sites in proteins[33], especially electrostatic interactions with the carboxy groups[34] from amino acids, like aspartate and glutamate[27], can give stable bonds (Figure 12). An example of such a mechanism is given by Reinhardt et al.[35] who observed that $Ca^{2+}$ binding to motifs in Fibrillin-1 (an extracellular matrix protein) stabilizes the structure against proteolytic degradation. In contrast to this result, in this study the addition of $Ca^{2+}$-ions led to an increase in degradation rate as was observed with type B gelatin microspheres. Here, the microspheres of the PBS+$CaCl_2$ group showed a significant higher weight loss than the PBS (pH = 5.72) group, whereas the pH during pretreatment of both groups was the same. An explanation is that with type B gelatin $Ca^{2+}$ crosslinks existed that stabilized the microparticle morphology but did not hamper microsphere degradation. The maintained microparticle structure possibly enhanced proteolytic degradation due to the larger surface area of these structures. The higher amount of carboxylic groups of type B gelatin (100-115 mM) when compared to type A gelatin (78-80 mM)[36] was responsible for the difference between the gelatin types, giving type B gelatin a better $Ca^{2+}$-binding capacity. Regarding this capacity, another explanation for the higher degradation rate is a difference in enzyme activity. Because $Ca^{2+}$ serves as a cofactor for interstitial collagenase[37-38], different authors have shown a reactivation of collagenase/gelatinase in the presence of 3.7-10 mM of free $Ca^{2+}$ [39-40]. Therefore if the gelatin also released $Ca^{2+}$ during proteolytic degradation it reactivated the enzyme, which would be more pronounced with the type B gelatin.

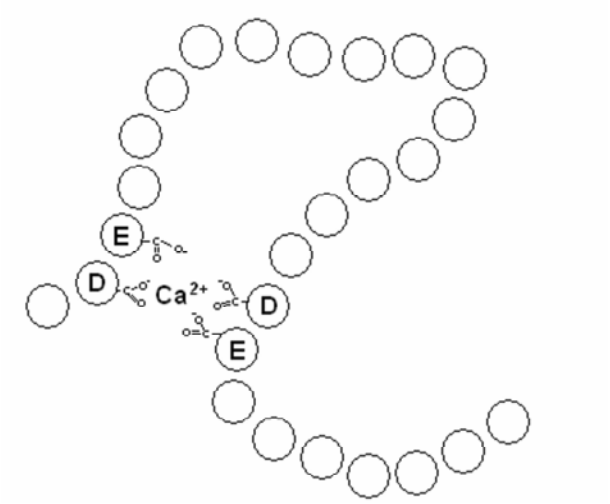



**Figure 12.** Folding of protein chains by interaction with $Ca^{2+}$

Comparison of the three types of gelatin in the supplementary degradation test revealed that in all pretreatment groups type A and type B gelatin microspheres degraded significantly faster than FG gelatin microspheres. The main difference between the two cell-culture tested gelatins and the FG gelatin is the bloom number that is significantly lower with the FG gelatin. The bloom number is a measure of gel strength of the gelatin[36] and is proportional to the average molecular weight of the chains. This implies that FG gelatin has a significant lower molecular weight and produces more gelatin chains per unit of microsphere surface than type A or type B gelatin. It therefore can be hypothesized that with type A and type B gelatin microspheres, enzymatic cleavage of a single "large" gelatin chain has a higher impact on microsphere degradation than with the FG gelatin, which results in a faster degradation.

## 5. Conclusion

The prepared gelatin microsphere CPCs were easy to handle with an initial setting time of less than 3 min. They possessed a compression strength of ± 30 MPa and a microsphere content of 43-44 vol%. *In vitro* degradation showed a modest pH decrease, a mechanical strength that remained constant in time and a small decrease in mass. SEM-investigation of the gelatin microsphere CPCs during the degradation test revealed little change in morphology, where separate gelatin microspheres showed complete degradation within the same time period. At the end of the degradation test, no increase in macroporosity was observed but even a clear densification of the composite. Parameters that contributed to this phenomenon were cement recristallization inside/onto the microspheres and a delayed gelatin degradation. From the supplementary microsphere degradation test it was concluded that the modest pH decrease present inside the cement is a factor in the delayed gelatin degradation. Also differences in degradation rate between different types of gelatin were observed. Overall, the gelatin microsphere CPC showed good handling properties, though degradation characteristics should be further investigated to generate a macroporous scaffold.

*Acknowledgements*

The authors would like to thank T.A. Holland and Z.S. Patel for technical assistance. Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) V.M. Goldberg, S. Stevenson, Natural history of autografts and allografts, Clin Orthop Relat Res. 225 (1987) 7-16

2) M.J. Imola, V, Sciarretta, V.L. Schramm, Skull base reconstruction, Curr Opin Otolaryngol Head Neck Surg. 11 (2003) 282-290

3) I. Khairoun, M.G. Boltong, F.C.M.. Driessens, J.A. Planell, Effect of calcium carbonate on clinical compliance of apatitic calcium phosphate bone cement, J Biomed Mater Res B: Appl Biomater. 38 (1997) 356-360

4) J.A. Jansen, E. Ooms, N. Verdonschot, J.G.C. Wolke, Injectable calcium phosphate cement for bone repair and implant fixation, Ortop Clin N Am. 26 (2005) 89-95

5) E.M. Ooms, J.G.C. Wolke, J.P.C.M. van der Waerden, J.A. Jansen, Trabecular bone response to injectable calcium phosphate (Ca-P) cement, J Biomed Mater Res Part A. 61 (2002) 9-18

6) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics, J Biomater Sci Polym Edn. 17(9) (2006)1057-1074

7) P.Q. Ruhé, E.L. Hedberg, N. Torio Paron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, RhBMP-2 release from injectable poly(DL-lactic-co-glycolic acid)/calcium-phosphate cement composites, J Bone Joint Surg Am. 85A S*uppl 3* (2003) 75-81

8) P.Q. Ruhé, O.C. Boerman, F.G. Russel, P.H.M. Spauwen, A.G. Mikos, J.A. Jansen, Controlled release of rhBMP-2 loaded poly(dl-lactic-co-glycolic acid)/calcium phosphate cement composites in vivo, J Control Release. 106(1-2) (2005) 162-171

9) C. Muzzarelli, R.A.A. Muzzarelli, Natural and artificial chitosan-inorganic composites, J Inorg Biochem. 92 (2002) 89-94

10) A. Berthold, K. Cremer, J. Kreuter, Collagen microparticles: carriers for glucocorticosteroids, Eur J Pharm Biopharm. 45(1) (1998) 23-29

11) D. Swatschek, W. Schatton, W. Muller, J. Kreuter, Microparticles derived from marine sponge collagen (SCMPs): preparation, characterization and suitability for dermal delivery of all-trans retinol, Eur J Pharm Biopharm. 54(2) (2002) 125-133

12) A. Bigi, E. Boanini, S. Panzavolta, N. Roveri, K. Rubini, Bonelike apatite growth on hydroxyapatite-gelatin sponges from simulated body fluid, J Biomed Mater Res Part A. 59 (2002) 709-714

13) T.A. Holland, Y. Tabata, A.G. Mikos, Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartillage tissue engineering, J Control Release. 101 (2005) 111-125

14) M.L. Bruschi, M.L.C. Cardoso, M.B. Lucchesi, M.P.D. Gremião, Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization, Int J Pharm. 264 (2003) 45-55

15) M. Yamamoto, Y. Ikada, Y. Tabata, Controlled release of growth factors based on biodegradation of gelatin hydrogel, J Biomater Sci Polym Edn. 12(1) (2001) 77-88

16) A. Muvaffak, I. Gurhan, N. Basirci, Prolonged cytotoxic effect of colchicine released from biodegradable microspheres, J Biomed Mater Res Part B: Appl Biomater. 71 (2004) 295-304

17) M.A. Vandelli, F. Rivasi, P. Guerra, F. Forni, R. Arletti, Gelatin microspheres crosslinked with D,L-glyceraldehyde as a potential drug delivery system: preparation, characterisation, in vitro and in vivo studies, Int J Pharm. 215 (2001) 175-184

18) H-C. Liang, W-H. Chang, K-J Lin, H-W. Sung, Genipin-crosslinked gelatin microspheres as a drug carier for intramuscular administration: *in vitro* and *in vivo* studies, J Biomed Mat Res Part A. 65 (2003) 271-282

19) I. Khairoun, D. Magne, O. Gauthier, J.M. Bouler, E. Aguado, G. Daculsi, P. Weiss, In vitro characterization and in vivo properties of a carbonated apatite bone cement, J Biomed Mater Res Part A. 60(4) (2002) :633-642

20) I Khairoun, M.G. Boltong, F.C.M. Driessens, J.A. Planell, Effect of calcium carbonate on clinical compliance of apatitic calcium phosphate bone cement, J Biomed Mater Res Part A. 39 (1997) 356-360

21) A. Bigi, G. Cojazzi, S. Panzavolta, K. Rubini, N. Roveri, Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking, Biomaterials. 22 (2001) 763-768

22) H.P. Stallmann, C. Faber, E.T. Slotema, D.M. Lyaruu, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11) from calcium phosphate bone substitutes, J Antimicrob Chemother. 52 (2003) 853-855

23) J. Ziegler, U. Mayr-Wohlfart, S. Kessler, D. Breitig, K-P. Günther, Adsorption and release properties of growth factors from biodegradable implants, J Biomed Mater Res Part A. 59 (2002) 422-428

24) E.A. Kremer, Y. Chen, K. Suzuki, H. Nagase, J.P. Gorski, Hydroxyapatite induces autolytic degradation and inactivation of matrix metalloprotease-1 and –3, J Bone Miner Res. 13(12) (1998) 1890-1902

25) D. Schomberg, M. Salzmann (editors), Enzyme Handbook, Springer-Verlang.(1991)

26) P. Monsan, G. Puzo, H. Mazarguil, Etude du mechanisme d'etablissement des liaisons glutaraldehyde-proteines, Biochimie. 57 (1975) 1281-1292

27) G.R. Tristam, The Proteins, New York: Academic Press Inc.(1953)

28) A. Simionescu, D. Sinionescu, R. Deac, Lysine-enhanced glutaraldehyde crosslinking of collagenous biomaterials, J Biomed Mater Res 25 (1991) 1494-1505

29) S-O. Molin, H. Nygren, L. Dolonius, A new method for the study of glutaraldehyde-induced crosslinking properties in proteins with special reference to the reaction with amino groups, J Histochem Cytochem. 26(5) (1978) 412-414

30) R.T. Morrison, R.N. Boyd, Organic Chemistry, vol3. Boston: Allyn and Bacon inc. (1973) 641-643

31) S. Matsuda, H. Iwata, S. Naomi, Y. Ikada, Bioadhesion of gelatin films crosslinked with glutaraldehyde, J Biomed Mater Res Part A 45 (1999) 20-27

32) S. Singh, K.V. Rama Rao, K. Venugopal, R. Manikandan, Alteration in dissolution characteristics of gelatin-containing formulations: A review of the problem, test methods, and solutions, Pharm Tech NA 26(4) (2002) 36-58

33) M.M. Yamashita, L. Wesson, G. Eisenman, D. Eisenberg, Where metal ions bind in proteins, Proc Natl Acad Sci USA. 87 (1990) 5648-5652

34) S. Peng, C. Wu, $Ca^{2+}$-induced complexation between thermally sensitive spherical poly(*N*-vinyl-caprolactam-*co*-sodium acrylate) microgels and linear gelatin chains in water, Polymer. 42 (2001) 7343-7347

35) D.P. Reinhardt, R.N. Ono, L.Y. Sakai, Calcium stabilizes fibrillin-1 against proteolytic degradation, J Biol Chem. 272(2) (1997) 1231-1236

36) Standard Methods for the Sampling and Testing of Gelatins. Gelatin Manifacturers Institue of America, Inc., 505 fifth Ave., Room 1015, New York, NY.

37) E. Morgunova, A. Tuuttila, U. Bergmann, M. Isupov, Y. Lindquist, G. Schneider, K. Tryggvason, Structure of Human Pro-Matrix Metalloproteinase-2: Activation Mechanism Revealed, Science. 284 (1999) 1667-1670

38) N. Borkakoti, Matrix metalloprotease inhibitors: design from structure, Biochem Soc Trans. 32(1) (2004) 17-20

39) J-O. Winberg, E. Berg, S.O. Kolset, L. Uhlin-Hansen, Calcium-induced activation and truncation of promatrix metalloprotease-9 linked to the core protein of chondroitin sulfate proteoglycans, Eur J Biochem. 270 (2003) 3996-4007

40) C. Sharpe, J.J. Robinson, Characterization of matrix metalloprotease activities induced in the sea urchin extraembryonic matrix, the hyaline layer, Biochem Cell Biol/ Biochim Biol Cell. 79(4) (2001) 461-468

# CHAPTER 5

## PORCINE GELATIN MICROSPHERE /CALCIUM PHOSPHATE CEMENT COMPOSITES: *IN VITRO* DEGRADATION AND PROTEIN RELEASE

*W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen*

Submitted to J Biomed Mater Res Part B: Appl Biomater. (2007)

## 1. Introduction

In the field of tissue engineering, gelatin microspheres are used by many researchers as a drug delivery system[1-3]. Characteristics that make these gelatin microspheres a good candidate for drug delivery are a very facile and controlled method of microparticle preparation[4-5], a biodegradability that can be tailored by crosslinking[6-7] and the possibility of obtaining a sustained release of drug / growth factor[8-9]. This sustained release is due to electrostatic binding of the drug with the gelatin. Next to that, gelatin microspheres degrade proteolytically and do not produce high amounts of acidic degradation products, unlike the PLGA/PLLA microspheres[10].

In a former experiment, gelatin microspheres were already introduced into an injectable calcium phosphate cement (CPC)[11]. CPC is a biocompatible and osteoconductive material that is commonly applied for the usage of bone filler in the field of dentistry, orthopedic and reconstructive surgery[12-14]. For tissue engineering applications, however, *in vivo* resorption of the bulk material and ingrowth of tissue is too slow[15]. Therefore, faster degrading gelatin microspheres (food-grade (FG), 5 wt%, 45 vol%) were introduced into the cement to produce a macroporous scaffold after proteolytic degradation of the microspheres. Results showed that though the mechanical and physical properties of the gelatin microsphere CPC were satisfactory, *in vitro* degradation of the composite showed no increase in macroporosity[11]. Parameters that contributed to this phenomenon were a delayed microsphere degradation inside the cement that was not influenced by the microsphere crosslinking density and recristallization of the cement onto/inside the microspheres, hampering the formation of interconnections between formed pores. In addition, a supplementary degradation test with gelatin microspheres showed that the type of gelatin also influenced the degradation properties as both porcine (type A) and bovine (type B) gelatin degraded faster than the applied FG gelatin, which was caused by the difference in molecular weight (Bloom number)[16] of the gelatin chains.

Because of the faster degradation, a gelatin type A microsphere CPC (GELA CPC) was formulated to obtain macroporosity into the cement. Also, with this gelatin a mechanically stable, injectable and setting 10 wt% gelatin microsphere CPC was synthesized that was hypothesized to produce a higher interconnectivity[17] of the spheres compared to the 5wt% FG gelatin microsphere CPC.

To increase *in vivo* resorption and bone ingrowth into the material, osteoinductive growth factors can be introduced into CPC. Release patterns, however, often show a marginal sustained release as the cement has a strong interaction with the loaded drug[18-19]. With a gelatin microsphere CPC, drug can be loaded inside the gelatin microspheres, thereby shielding the drug from the surrounding cement. To investigate drug/protein release from GELA CPCs, gelatin type A (GELA) microspheres (pI = 7.0-9.0) can be used to electrostatically bind acidic model proteins like bovine serum albumin (BSA, pI =5.0) in an *in vitro* release study[20].

In view of the above mentioned, the goal of the current study was to investigate the effect of the inclusion of GELA microspheres in CPC on *in vitro* degradation properties and drug/protein release characteristics of the cement. Therefore, a 12 week degradation test was performed in proteolytic

medium and samples were assayed on mass, compression strength and morphology. Release characteristics of the composites were determined by loading the microspheres with BSA using two different methods, after which BSA concentration in the release medium was measured by reversed-phase (RP)-HPLC. Finally, the structure of the released proteins was assayed using SDS-PAGE.

## 2. Materials and Methods

### *2.1. Materials*

Gelatin (Powder, type A, IEP 7.0-9.0, Acros Organics, Geel, Belgium) was used for the preparation of the microspheres. Olive oil (Acros Organics, Geel, Belgium) and acetone (HPLC grade, Labscan ltd., Dublin, Ireland) were used as substrate during microsphere preparation. Glutaraldehyde (25wt% solution, EM-grade, Merck, Darmstadt, Germany) was applied as crosslinker. The calcium phosphate cement (Calcibon®, Biomet Merck, Darmstadt, Germany) consisted of 61% α-TCP, 26% $CaHPO_4$, 10% $CaCO_3$ and 3% precipitated HA. The cement liquid applied was a 1% aqueous solution of $Na_2HPO_4$.

### *2.2. Methods*

#### *2.2.1. Preparation GELA microspheres*

2.5 g of gelatin was dissolved in 25 ml $ddH_2O$ at an elevated temperature (30 min at 60°C). The resulting clear solution was added slowly (10 ml pipette) to a 250 ml three-necked round bottom flask containing 125 ml olive oil while stirring at 500 rpm (PTFE upper stirrer). After the gelatin solution was added to the oil, stirring was continued and the round bottom flask was put in an ice bath so that the temperature of the oil phase was kept at 4°C. After 30 min, 50 ml of chilled acetone (4°C) and glutaraldehyde (0.5 ml = 6.25 mM) was added slowly. The solution was stirred and cooled for another 1h. Microspheres were collected by filtration (D3, Schott Duran, Mainz, Germany) and washed several times with acetone (approx. 1 l) to remove residual olive oil. After that, they were stored in a drying chamber until further use.

#### *2.2.2. Preparation GELA CPC*

10 wt% gelatin microsphere/calcium phosphate cement composites were prepared. To generate an equal distribution of microspheres inside the cement, GELA microspheres were swollen first before cement was added. Therefore, 100 mg of microspheres were put in a 2 ml plastic syringe; subsequently 560µl $ddH_2O$ was added after which the syringe was stirred vigorously for 15 s (Silamat® mixing apparatus, Vivadent, Schaan, Liechtenstein). To the resulting swollen microspheres, 900 mg of cement was added and the mixture was stirred for 15 s. Then 260 µl 1% $Na_2HPO_4$ solution was added and the contents were stirred again for 15 s. The acquired paste was injected into a PTFE mould (cylinders 4.5

x 9 mm), after which the samples were left to set at room temperature for 24 hr and stored in a drying chamber.

*2.2.3. Morphology analysis & size distribution*

The morphology of dry GELA microspheres and GELA CPC was visualized by scanning electron microscopy (SEM) (JEOL 6400-LINK AN 10000 at 10 kV). Swollen spheres were suspended in demineralised water ($ddH_2O$) for 1 day before pictures were taken with an optical microscope (Leica DMRBE microscope, Leica Microsystems AG, Wetzlar, Germany). The size distribution of dry and swollen spheres was determined using digital imaging software (Leica Qwin, Leica Microsystems AG, Wetzlar, Germany).

*2.2.4. Setting time & cohesion properties*

Setting time and cohesion properties of the GELA CPC were determined as described previously[21]. In brief, initial and final setting times were assessed using custom available Gillmore needles. For this, a bronze block containing 6 holes (6 mm in diameter, 12 mm in height) was used as mould and placed in a water bath at 37˚C. Samples were mixed and injected in a retrograde fashion, after which the setting time was determined (n = 3). For determination of the cohesion properties, samples were injected into Ringer's solution at 37°C. During cement setting it was observed whether the paste retained its original configuration. Every anomaly was recorded.

*2.2.5. Porosity*

Of the GELA CPC, the total and macroporosity was determined. Macroporosity is porosity generated by (the degradation of) gelatin microspheres. The total porosity is the macroporosity plus the original microporosity of the cement. When the gelatin microspheres are not degraded, the macroporosity corresponds to the volume% of microspheres present inside the cement initially.

To measure these parameters, both GELA CPC samples and (microporous) CPC samples of a known volume were placed in an oven at 650°C for 2 h. After burning out the gelatin/moisture, samples were weighed and equation 1 and 2 were used for the derivation of the total porosity and the macroporosity. Samples were taken threefold (n=3).

$$\varepsilon_{tot} = (1 - \frac{m_{macro/micro}}{V * \rho_{HAP}}) * 100\% \qquad \textit{Equation 1}$$

$$\varepsilon_{macro} = (1 - \frac{m_{macro}}{m_{micro}}) * 100\% \qquad \textit{Equation 2}$$

*Legend:* $\varepsilon_{tot}$ = total porosity (%), $\varepsilon_{macro}$ = macroporosity (%), $m_{macro}$ = average mass macroporous sample (after burning out gelatin) (g, n=3), $m_{micro}$ = average mass microporous CPC sample (g, n=3), V = volume sample ($cm^3$), $\rho_{HAP}$ = density hydroxy apatite ($g/cm^3$)

*2.2.6. Degradation assay*

For the degradation assay GELA CPC samples were placed in 3 ml of phosphate-buffered saline (PBS, pH 7.4) containing 50µg/ml gentamycin and 373ng/ml collagenase 1A (Sigma-Aldrich, St. Louis, USA) and incubated at 37˚C in a water bath on a shaker table (70 rpm) for a total of 12 weeks (84 days). Every 3-4 days sample buffer was renewed; at day 14, 28, 42, 63 and 84, 5 specimens of the different cement formulations were subjected to analysis as summarized below. CPC samples were used as reference.

Mass loss quantification

Samples were lyophilized overnight before the mass was measured. The mass loss of the samples was calculated using Equation 3.

$$R_L = \frac{M_0 - M_n}{M_0} * 100\% \qquad \textit{Equation 3}$$

*Legend:* $R_L$ = mass loss sample on t = n (%), $M_0$ = mass sample on t = 0 (g), $M_n$ = mass sample on t = n (g)

Mechanical characteristics

Samples were placed in a testing bench (858 MiniBionixII, MTS Corp., Eden Prairie, MN, USA) and compression strength and E-modulus along the height of the specimens was measured at 0.5 mm/min crosshead speed.

Morphology

Morphology of the samples was determined using SEM. Pictures at a magnification of 100x were taken to visualize composite/microsphere degradation at approximately 1 mm from the outside of the sample.

*2.2.7. Release study*

Preparation BSA-loaded gelatin microsphere CPC

GELA CPC was loaded with BSA using two different methods. The first method, where the composites were made directly after adding the BSA to the microspheres, is referred to as instant loading. The second method, where the BSA was allowed to settle inside the microspheres, we refer to as prolonged loading. Prolonged loading was applied to achieve a stronger electrostatic interaction between the BSA and the gelatin, as is normally performed in literature[8-9].

For both methods, samples were loaded with BSA by adding 560µl of a 2.1% BSA solution to 100mg of GELA microspheres inside a 2ml plastic syringe. With instant loading, the microspheres and BSA solution were mixed for 15s with the Silamat mixing apparatus. Then, 900 mg of cement was added and gelatin microsphere composites were prepared as described in "preparation GELA CPC". With prolonged loading, the microspheres and the BSA solution were mixed for 15s and stored at 4°C for 24 h. Subsequently, cement was added and composites were prepared. As 6-7 samples were extracted

from each syringe, the calculated amount of BSA per sample with both methods was 1.5 mg, which was based on a homogeneous distribution (wt%) of microspheres inside the composite paste.

Furthermore; to visualize BSA loading with both loading mechanisms, in a separate experiment fluorochrome labeled BSA (Alexa Fluor® 488, Molecular Probes, OR, USA) was added to the BSA solution in a ratio of 1/1000 and loaded onto the microspheres. After BSA loading, these microspheres were dispersed in water and analyzed by fluorescence light microscopy (Leica DMRBE microscope, Leica Microsystems AG, Wetzlar, Germany) and confocal laser scanning microscopy (CLSM, MRC 1000, Biorad, CA, USA).

BSA release assay

During the release test, 3 samples of each formulation were put in release medium ($ddH_2O$ + 373 ng/ml collagenase 1A) in 2ml Eppendorf tubes and incubated in a water bath (37ºC) on a shaker table (70 rpm) for 9 weeks. Initially 1.8 ml of medium was added, then samples of 200µl were taken at t = 0.5, 1.0, 2.0 and 24 h. For the following weeks, sample medium was refreshed with 1.0 ml every 3-4 days. BSA concentration of the sample medium at the different time points was measured and quantified by reversed-phase high performance liquid chromatography (RP-HPLC) using an Atlantis® RP-HPLC column (Waters Corp., Milford, MA, USA) a L-2130 HPLC pump (Hitachi, Tokyo, Japan) and a L-2400 UV detector (Hitachi) which was set at 280 nm. Furthermore, during measurement a 40/60 acetonitrile/water mixture containing 10% 0.25 M formic acid was used as mobile phase at an isocratic flow rate of 0.70 ml/min and column temperature of 35°C. For determination of the BSA concentration a primary calibration curve was prepared. GELA CPC samples without BSA were used as reference as despite the difference in UV absorption maximum between gelatin and BSA (gelatin = 230 nm[22], BSA = 280 nm) and absence of a UV-absorption band at 280 nm for the CPC a pilot study showed some signals from the GELA CPC in HPLC (Figure 1).

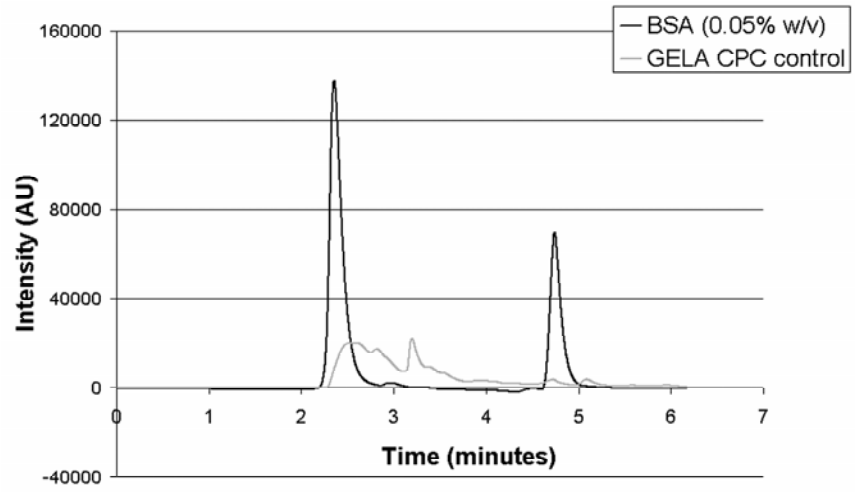



**Figure 1.** RP-HPLC chromatograms of bovine serum albumin (0.05 % w/v in water) and products from GELA CPC control after 3 days incubation

Structure analysis

For further investigation of the BSA-release, the molecular weight of the released proteins within the first weeks was analyzed using sodium dodecyl sulphate poly (acryl amide) gel electroforesis (SDS-

PAGE). In brief, 0.5 ml of the sample medium was lyophilized and concentrated (20x). After that, samples were separated on a 10% poly acryl amide (PAA) gel and detected using a Chroomassie-blue staining. As reference non-loaded scaffolds, a 0.01% BSA-solution, a gelatin solution and a broad range SDS standard (Biorad®, Hercules, CA, USA) were used.

*2.2.8. Statistical analysis*

Data were arranged as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat®.

## 3. Results

### *3.1. Preparation microspheres / handling properties*

SEM-micrographs of the prepared microspheres are depicted in Figure 2A. The microspheres showed a spherical appearance as well as imprints of other spheres at their surface. The size distribution graph (Figure 3A) showed that 51% of the dry spheres were in the range of 0-10 μm, and after swelling a clear shift was observed to higher microsphere sizes. The size of the swollen spheres was more than doubled, with an average of 37.4 μm (Table 1). Regarding the vol% of the swollen spheres (Figure 3B), the large amount of small spheres (<10 μm) only contributed to 1% of the total volume, whereas the few larger spheres > 150 μm contributed to 50%.

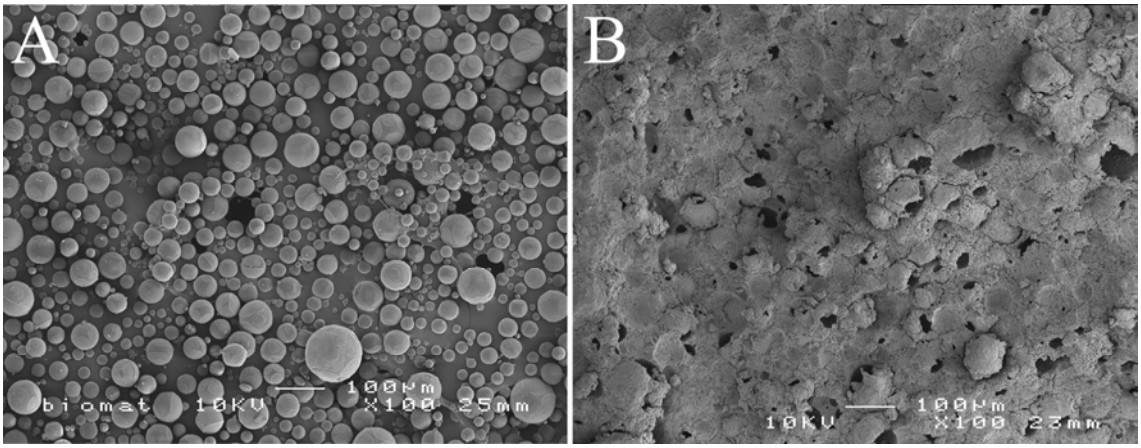



**Figure 2.** Determination morphology by SEM; A = Dry microspheres (original magnification 100x), B = Microspheres in cement after 1 day cement setting (original magnification 100x)

Parameters of the cement / scaffold after setting are also given in Table 1. The initial setting time of the GELA CPC was two times the setting time of the CPC[21]. No final setting time was measured due to the high water content and compressibility of the gelatin microspheres[11]. During the cohesion test the GELA CPC stayed in a solid shape, though some powder was released upon injection. After hardening, the scaffolds exhibited compression strengths of ±19 MPa which was significant lower than

the CPC samples ($p<0.01$). The gelatin microsphere content (macroporosity) corresponded to 57.5vol%. SEM-micrographs directly after hardening (Figure 2B) showed a tight packing of microspheres, where most spheres were broken and covered by a layer of calcium phosphate precipitate.

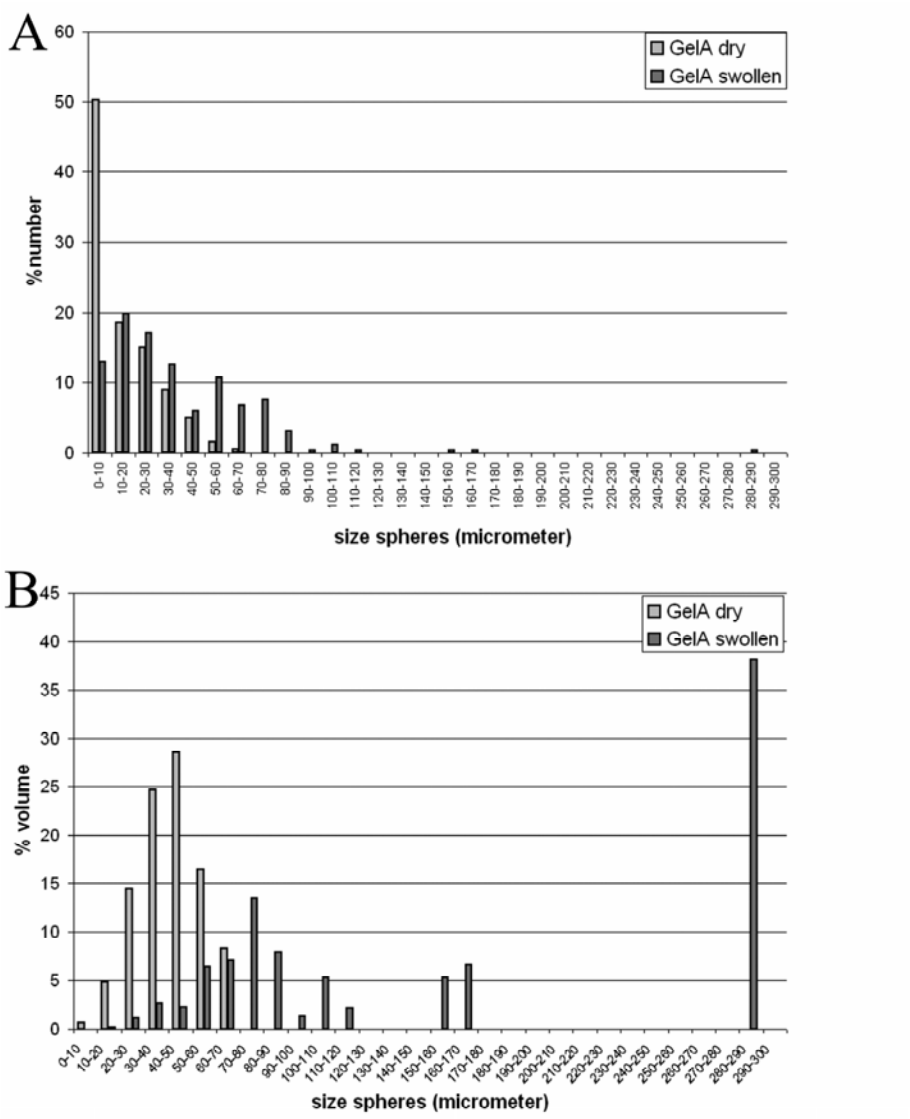



**Figure 3.** Size distribution graphs of dry and swollen gelatin microspheres; A = Number average distribution, B = Volume average distribution, group size n=200 (dry)/n=263 (swollen)

**Table 1.** Parameters GELA CPC and CPC

| Parameter | GELA CPC | CPC |
|---|---|---|
| Average microsphere size (μm) | 15.48 ± 13.79 (dry) | - |
| | 37.39 ± 31.07 (swollen) | |
| Initial setting time (s) | ± 210 | 108.3 ± 5.8 |
| Porosity (%) | 74.8 ± 0.6 | 45.52 ± 1.26 |
| Macroporosity/ microsphere content (vol%) | 57.5 | - |
| Compression strength (MPa) | 18.5 ± 4.5 | 48.2 ± 7.8 |
| Compression E-modulus (MPa) | 905.8 ± 306.2 | 3140.5 ± 351.8 |
| Weight sample, 4.5*10 mm (mg) | 127.6 ± 4.3 | 262.7 ± 1.26 |

### *3.2. Degradation assay*

The results of the degradation assay are given in Figure 4-6. The mass of the GELA CPC showed an almost linear decrease in time. After 84 days a total mass loss of 5% was observed. CPC only showed an increase in mass and already after 14 days significant differences with the GELA CPC were observed (14d: $p<0.01$, 84d: $p<0.001$).

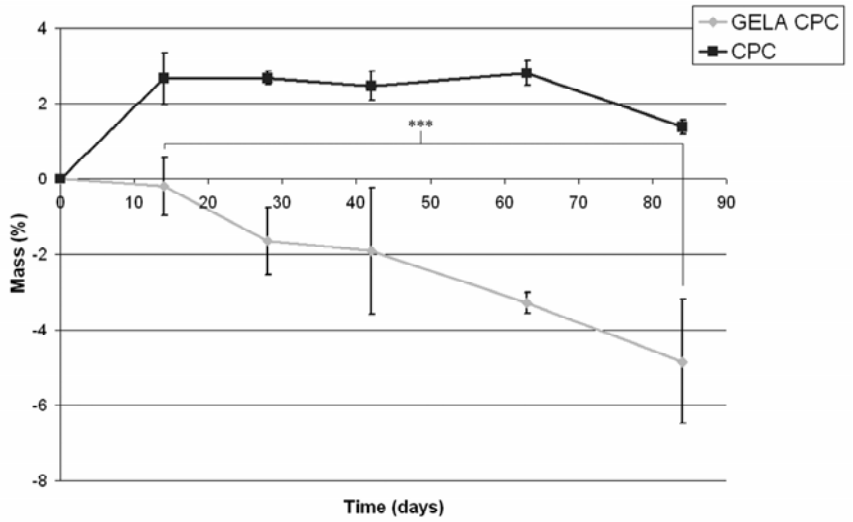



**Figure 4.** Development mass GELA CPC and CPC as function of degradation time, ***=$p<0.001$

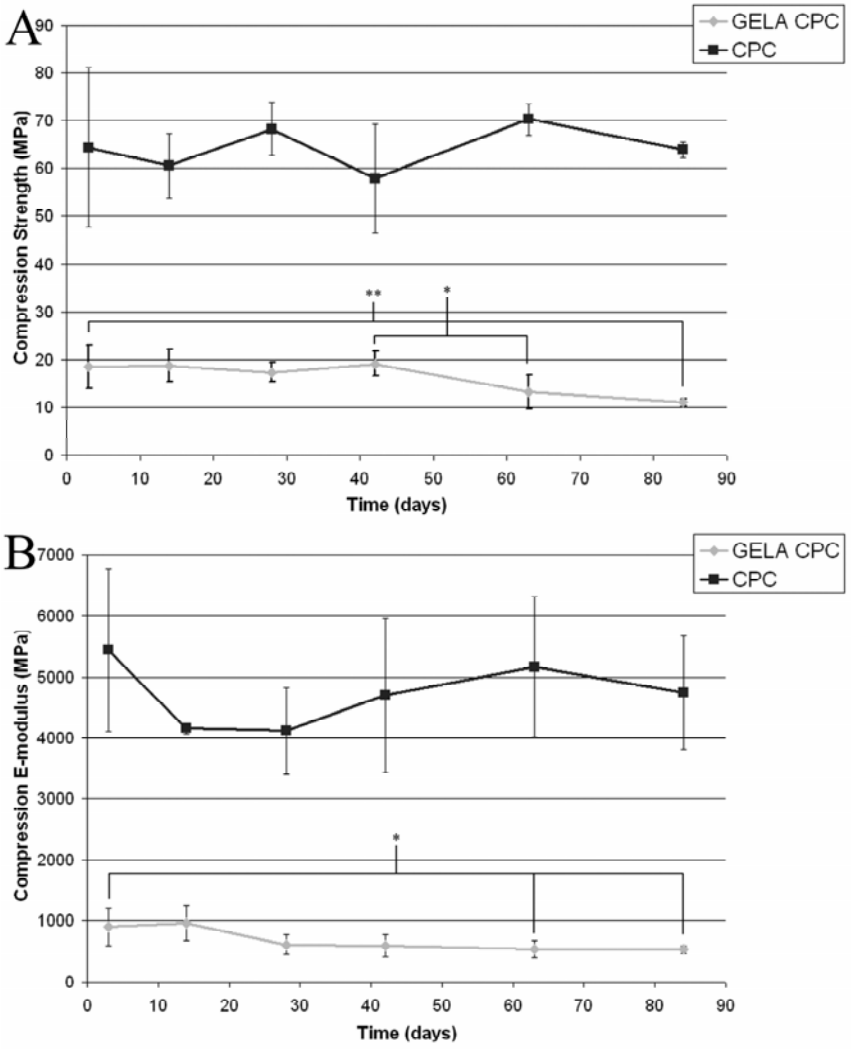



**Figure 5.** Mechanical properties GELA CPC and CPC as function of degradation time; A = Compression strength, B = E-modulus, ** = $p<0.01$, * = $p<0.05$

The compression strength of the GELA CPC (Figure 5A) was constant at 19 MPa till 42 days, after which a significant decrease was observed to 11 MPa at 84 days. The E-modulus (Figure 5B) also showed a significant decrease from 900 MPa at 3 days to 500 MPa after 84 days. The compression strength/ E-modulus of the CPC remained constant during the degradation test.

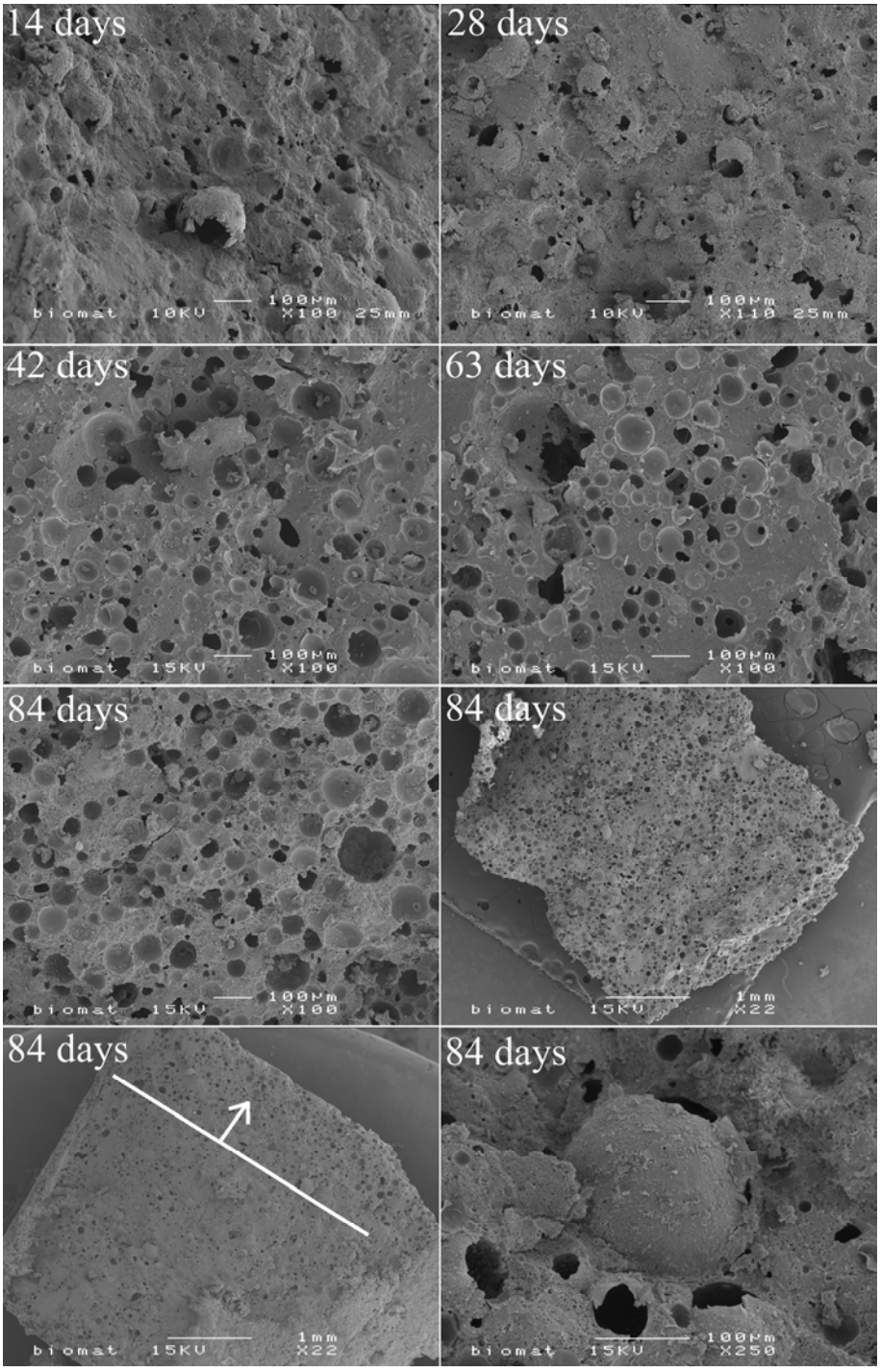



**Figure 6.** Morphology GEL A CPC with SEM at 1 mm from the outside radius at t = 14, 28, 42, 63 and 84 days (original magnification 100x), overview of samples after 84 days (original magnification 22x) and close-up picture of gelatin microspheres at the inner part of the composite (original magnification 250x)

SEM-micrographs of the GELA CPC at 1 mm below the surface showed a structure of broken microspheres inside the cement till t = 42 days (Figure 6). Starting from 42 days, less microsphere structures were present and a clear increase in porosity was observed. Also interconnections between

the pores were visible. At 84 days the gelatin spheres at this location appeared to be totally degraded. However, at a lower magnification, at this time point still gelatin microspheres were visible in all the samples at the inner part of the composite, while at the outside gelatin was completely degraded.

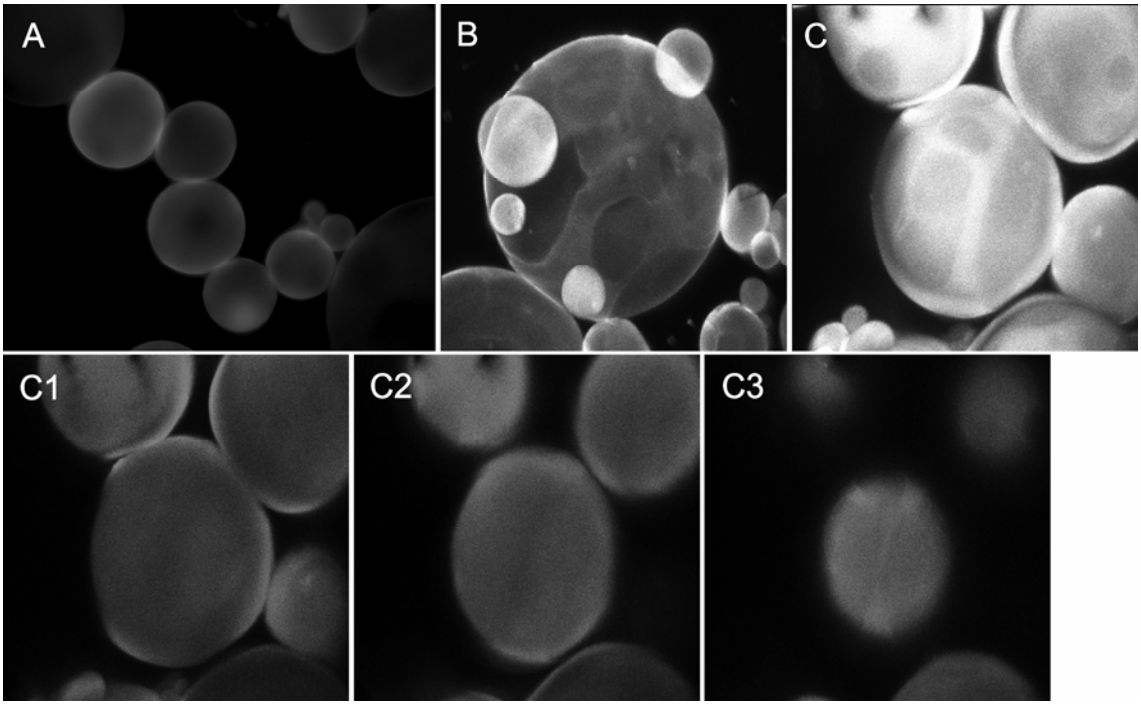



**Figure 7.** Fluorescence microscopy (A) and stacked CLSM micrographs (B+C) of fluorochrome labeled-BSA loaded gelatin microspheres, C1-C3 are intersections of microspheres shown in figure C at different heights (original magnification 40x)

### *3.3. Release study*

Pictures of fluorochrome labeled BSA-loaded microspheres are depicted in Figure 7. The labeled BSA was adsorbed by the gelatin microspheres that showed a high concentration of fluorescent label. The surface structure of the microspheres was clearly visible in CLSM and label intensity differed between the microspheres irrespective of the microsphere size. Furthermore, BSA was present at the surface and in the middle of the spheres, as is clear from the CLSM micrographs at different heights, but distribution varied greatly among the microspheres and no difference between both loading mechanisms was obtained.

The % release of BSA per time interval as well as the cumulative release from the GELA CPC is given in Figure 8 as a function of incubation time. The cumulative release of water solvable products from the GELA CPC (reference samples) is also given; however, because these products cannot be calibrated, the release is expressed as function of the peak area. For both loading methods almost no initial burst of BSA was observed. Between 1-7 days a high standard deviation was obtained due to the concomitant release of high amounts of water-solvable products from the GELA CPC that overlapped with the BSA signals. However, especially with the instant loaded scaffolds a substantial release of 10-15% of BSA was observed. In the second and the third week significant higher amounts of BSA were

released from the instant loaded scaffolds compared to the prolonged loaded scaffolds. After 21 days the prolonged loaded scaffolds showed a sustained release of 1.5-2% per week, where the instant loaded scaffolds showed a further decrease of released BSA. In week 8 and 9 significant higher amounts of BSA were released from the prolonged loaded scaffolds. The total release efficiencies after 63 days were 42.8± 9.1% (instant) and 26.1 ± 12.6% (prolonged).

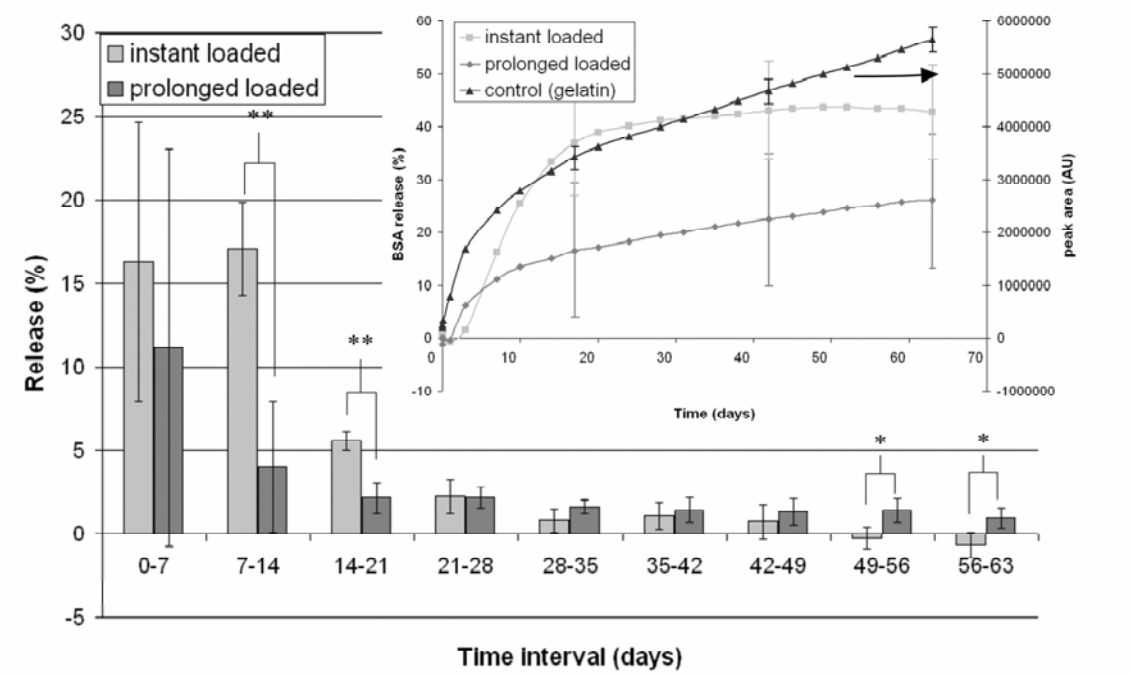



**Figure 8.** BSA release per time interval from instant and prolonged loaded GELA CPC, cumulative BSA release from instant and prolonged loaded GELA CPC (inlay, left axis) and cumulative gelatin release from control samples as calculated from peak area in AU (non-loaded GELA CPC, inlay, right axis) , ** = $p<0.01$, * = $p<0.05$

Results of the SDS-PAGE measurement are given in Figure 9. The chromatogram at 3 days showed that the medium of the instant loaded scaffolds exhibited a strong signal at the height of BSA, whereas with the prolonged loaded scaffolds only a very weak BSA signal was present. However, this sample did show some extra bands at higher molecular weight that were also visible with both instant/prolonged loaded scaffolds at day 7. At 17 days the same signals were still present with the instant loaded scaffolds, while only low molecular weight products were observed with the prolonged loaded scaffolds. The GELA CPC control samples did not show specific signals at a certain molecular weight but a continuous band from high to low molecular weight at t = 3 and 7 days, which was also visible with the BSA loaded samples and can be appointed to gelatin degradation products. At t = 17 days the chromatogram of the control samples corresponded with the prolonged loaded scaffolds and some signal was observed at molecular weights < 21.5 kDa.

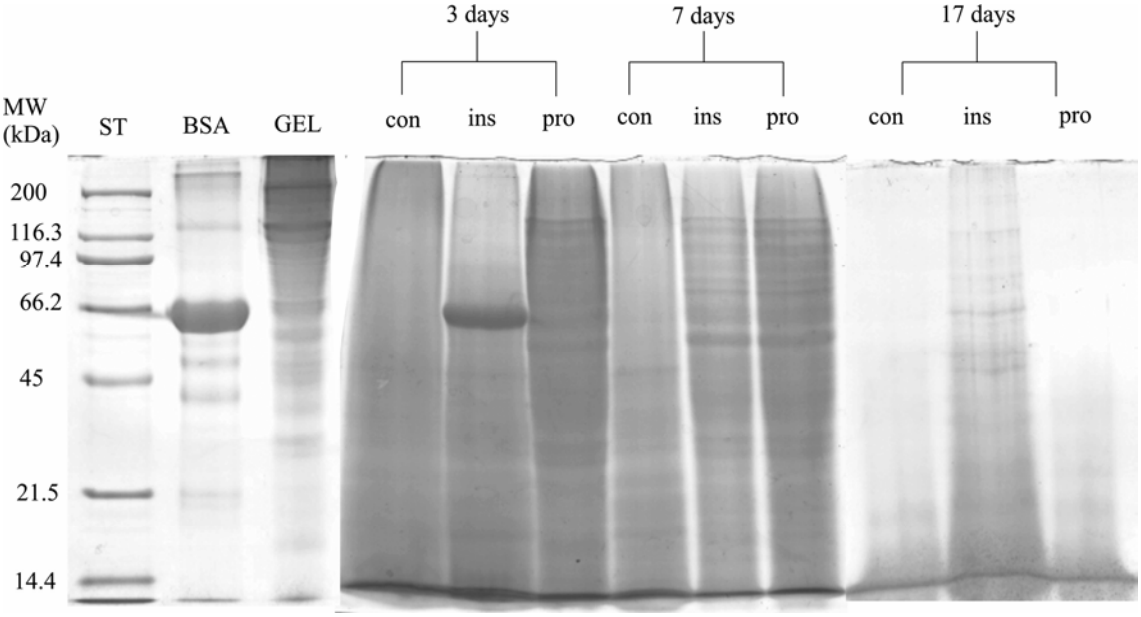



**Figure 9.** SDS-PAGE chromatograms of BSA loaded GELA CPC at t = 3, 7 and 17 days. Legend: ST = standard, GEL = gelatin control, CON = control (non-loaded GELA CPC), INS = instant loaded GELA CPC, PRO = prolonged loaded GELA CPC.

## 4. Discussion

The aim of this experiment was to synthesize a gelatin type A microsphere / calcium phosphate cement composite (GELA CPC) for bone tissue engineering purposes. The addition of gelatin microspheres was hypothesized to introduce macroporosity into the CPC, increase biodegradability and optimize the release of growth factors that are often applied for bone remodeling. To test this hypothesis, the composite was subjected to a degradation test in proteolytic medium to investigate the degradation properties/mechanism and formation of macroporosity by gelatin microsphere erosion. Secondly, protein/drug release properties of the GELA CPC were investigated by adding a model protein (BSA) to the microspheres using two different loading methods.

As mentioned in the introduction, gelatin type A was used in the current study because this material exhibited a faster *in vitro* resorption rate than the food-grade (FG) gelatin in a previous experiment[11]. Next to that, a fully injectable and setting 10 wt% (57.5 vol%) GELA CPC was synthesized that was hypothesized to exhibit a better interconnectivity of the microspheres compared to the 5wt% FG gelatin CPC. With FG gelatin microspheres, a 5wt% composite was maximum regarding setting properties, whereas with GELA microspheres this was 10wt% and therefore a higher amount of microspheres could be introduced. The setting process of these composites, when compared to the original cement, is delayed by leakage of water from the swollen microspheres[23]. Considering this, the difference between both groups can be due to the larger size of the GELA microspheres that exhibit a smaller interface with the surrounding cement than an identical vol% of smaller FG gelatin microspheres. The occurrence of a small amount of swollen microspheres > 100 μm (Figure 3) also could be favorable for bone ingrowth as multiple authors[17, 24-26] state that for this process pores are required with a minimal size of 100-200μm. However, the addition of too many of such pores can

decrease the mechanical properties of the composite as in literature[21,27-28] cements with large and irregular shaped pores show less mechanical properties than cements with smaller and regular shaped pores.

From the degradation test it was concluded that the GELA CPC showed a significant decrease in mass and compression strength after 84 days in proteolytic medium, whereas CPC was mechanically stable and experienced an increase in mass that was caused by PBS precipitation[11]. SEM-investigation also showed a clear degradation of the microspheres in time. It was observed that the microspheres first degraded at the outside of the implant and degradation gradually proceeded to the inner part. This process has to be considered as surface erosion[29], though in a previous degradation experiment separate microspheres degraded in a bulk erosion fashion showing gradual deformations in microsphere structure and higher degradation rates[11]. A reason for the surface-like erosion mechanism is that the collagenase from the medium did not enter the middle of the composite due to binding of the protein to the cement[18-19] or even deactivation[30]. When the microspheres degraded they left an interconnected structure of macropores that enabled diffusion of the enzyme into the composite without binding to the cement. The overall compression strength decreased while after gelatin degradation a weaker skeleton of calcium-deficient carbonate apatite remained. The surface-like erosion mechanism observed in this experiment, proves that gelatin microspheres can be applied to produce macroporosity inside a calcium phosphate cement. Explanations for the improved degradation of the GELA CPC when compared to the FG gelatin CPC[11] are indeed a better interconnectivity of the microspheres and a faster degrading gelatin. Similar to the previous degradation experiment, when incorporated inside the cement, the GELA microspheres showed a layer of calcium phosphate precipitate on the surface[31] that could hamper interconnectivity of the pores. However, due to the higher vol% of microspheres inside the GELA CPC, interconnections were still present.

The surface-like erosion mechanism observed for the GELA CPC is not unfavorable for bone tissue engineering purposes. In previous *in vivo* experiments with macroporous cements[32-33] it was shown that with these osteoconductive implants new bone ingrowth proceeded from the outside of the implant to the inside. So when macroporosity is first present at the superficial regions it can guide bone ingrowth, while in the middle of the composite strength is retained as microspheres are not degraded yet. Upon the addition of poly(lactic-*co*-glycolic) acid (PLGA) microspheres into an injectable CPC in a previous *in vitro* degradation experiment[21] bulk erosion of the microspheres was observed. This implicates that when the same mechanism occurs *in vivo*, microsphere erosion and therefore the decrease in compression strength occurs concomitantly though the whole composite, leaving a less stable implant as bone is not present in the middle. However, extrapolations of the obtained *in vitro* degradation mechanisms for both GELA CPC and PLGA CPC to *in vivo* behavior is complicated due to different conditions with respect to fluid flow, consistency of medium and sources of gelatin degrading enzymes[34].

Another advantage of GELA CPC over the PLGA microsphere containing cements is the drug loading mechanism. With polymers such as polylactic acid (PLA) and PLGA drugs are introduced during the microparticle preparation method, which leads to losses of drug and even inactivation[35]. Because gelatin is a hydrogel, an aqueous protein/drug solution can be added to dry microspheres just before they are mixed with the cement and during swelling the protein/drug will be electrostatically adsorbed by the microspheres as was observed by CLSM. With this method almost all the protein/drug is inside the composite, and inactivation by chemical solvents is non-existant. However, because loading efficiencies of gelatin microspheres are not optimal [20] and release patterns from gelatin microspheres often show a high burst release[9], some protein/drug will be released into the calcium phosphate cement as water diffuses out of the microspheres during cement mixing. This is not beneficial because the cement has a strong affinity with proteins/drugs, and possibly only a small amount will be released when included inside the cement as was observed by multiple authors[18-19, 36]. High release efficiencies are important while growth factors that are used for tissue engineering applications are expensive and can rise the costs for a future application[37].

The release patterns that were observed with the HPLC analysis showed that instant loaded GELA CPC scaffolds exhibited a total BSA release of more than 40% within 21 days. This was significantly higher than *in vitro* rhBMP-2 or BSA release from PLGA microsphere CPC composites and plain CPC that showed a 3-15% release within 28 or 42 days[38-39]. A reason for this improvement could be the porous structure of swollen gelatin microspheres, which is permeable for smaller molecules and facilitates BSA diffusion out of the scaffold. However, release efficiencies with this cement are very much dependent on the type of protein or drug used[28, 40] and the pH of the surrounding medium[41]. During degradation experiments of PLGA microsphere CPC the pH was found to drop substantially in time during microsphere degradation[21] whereas with gelatin microspheres no additional decrease in pH was observed[11]. Secondly, from SDS-page measurements we can conclude that in this study also a high release of (water-solvable) gelatin chains occurred within the first week. The signal observed for the GELA CPC controls in RP-HPLC (Figure 1) therefore is also likely caused by gelatin degradation products, although unmodified gelatin only has a very weak absorption at 280 nm[22]. This concomitant gelatin release influenced the release of BSA. A study of Ruhé et al[42] showed that coating the cement with BSA increased the release efficiency of added BMP-2, which implicates that the protein binding capacity of the cement can be saturated at a certain concentration resulting in “weaker” protein-protein interactions. The bulk of gelatin chains that was released within the first days therefore possibly saturated the cement. After that, the remaining BSA diffused more easily out of the scaffolds rendering the delayed release as was observed with both loading methods at 3-7 days.

From 7-21 days the instant loaded scaffolds showed a significantly higher BSA release than the prolonged loaded scaffolds. This can be explained by a weaker bonding between the gelatin and the BSA with the instant loaded scaffolds. In more detail, according to literature[20] proteins that are adsorbed to gelatin microspheres will release during degradation of the gelatin. As mentioned in the

materials and methods section, to achieve a strong electrostatic interaction between the protein and the gelatin, the protein normally is allowed to settle inside/onto the gelatin microspheres for a certain period before they are put in release medium[8-9]. A similar procedure was done with the prolonged loaded scaffolds for 24h but not with the instant loaded scaffolds. Therefore with this loading method electrostatic interactions between the BSA and the gelatin were weaker and the BSA diffused faster out of the GELA CPC composites. Furthermore, the sustained release that was observed with the prolonged loaded scaffolds after 21 days can be attributed to the degradation of the gelatin microspheres.

In SDS-page a very strong band of unaltered BSA was observed after 3 days in release medium with the instant loaded scaffolds, while for the prolonged loaded scaffolds and also for later time points the BSA signal was much weaker and bands of higher molecular weight were observed. This result indicates that indeed the BSA in the instant loaded scaffolds was more loosely bonded to the gelatin than in the prolonged loaded scaffolds. The occurrence of the signals at higher molecular weight implies that BSA/gelatin complexes were formed due to the electrostatic interaction between the protein structures[20]. After 17 days these "complexes" were also observed with the instant loaded scaffolds, though with the prolonged loaded scaffolds only low molecular weight products were seen. This was partially due to a decrease in concentration below the detection limit. Degradation of the BSA in time is also a possibility because in literature[40] degradation of growth factors was observed with comparable calcium phosphate ceramics.

Interpreting the results of the release and degradation assay, figure 10 gives a hypothetic model of the release mechanism. From the beginning a scaffold was obtained in which the BSA was distributed into the dry gelatin spheres, but also was present inside the surrounding calcium phosphate cement (A). After placing the composite into the release medium, the gelatin microspheres were swollen and showed a high release of gelatin chains and a delayed release of BSA into the medium. With the instant loaded scaffolds the release consisted of a high amount of unaltered BSA (B), while with the prolonged loaded scaffolds most likely also complexes between the gelatin and BSA were present as they bind electrostatically[8, 9, 20] (C). After releasing most of the loosely bonded protein, the release was continued due to the proteolytic degradation of the gelatin spheres[20]. Here, the gelatin started to erode from the outside to the inside and especially with the prolonged loaded scaffolds small amounts of BSA or BSA/gelatin complexes were released into the medium (D,E). Furthermore, it can be hypothesized that after gelatin microsphere degradation, but also in an earlier stage, a small amount of the BSA that is (still) trapped inside the cement, diffuses out of the cement skeleton and gives a slow release in time (F) as was observed by multiple authors[18-19, 36, 39].

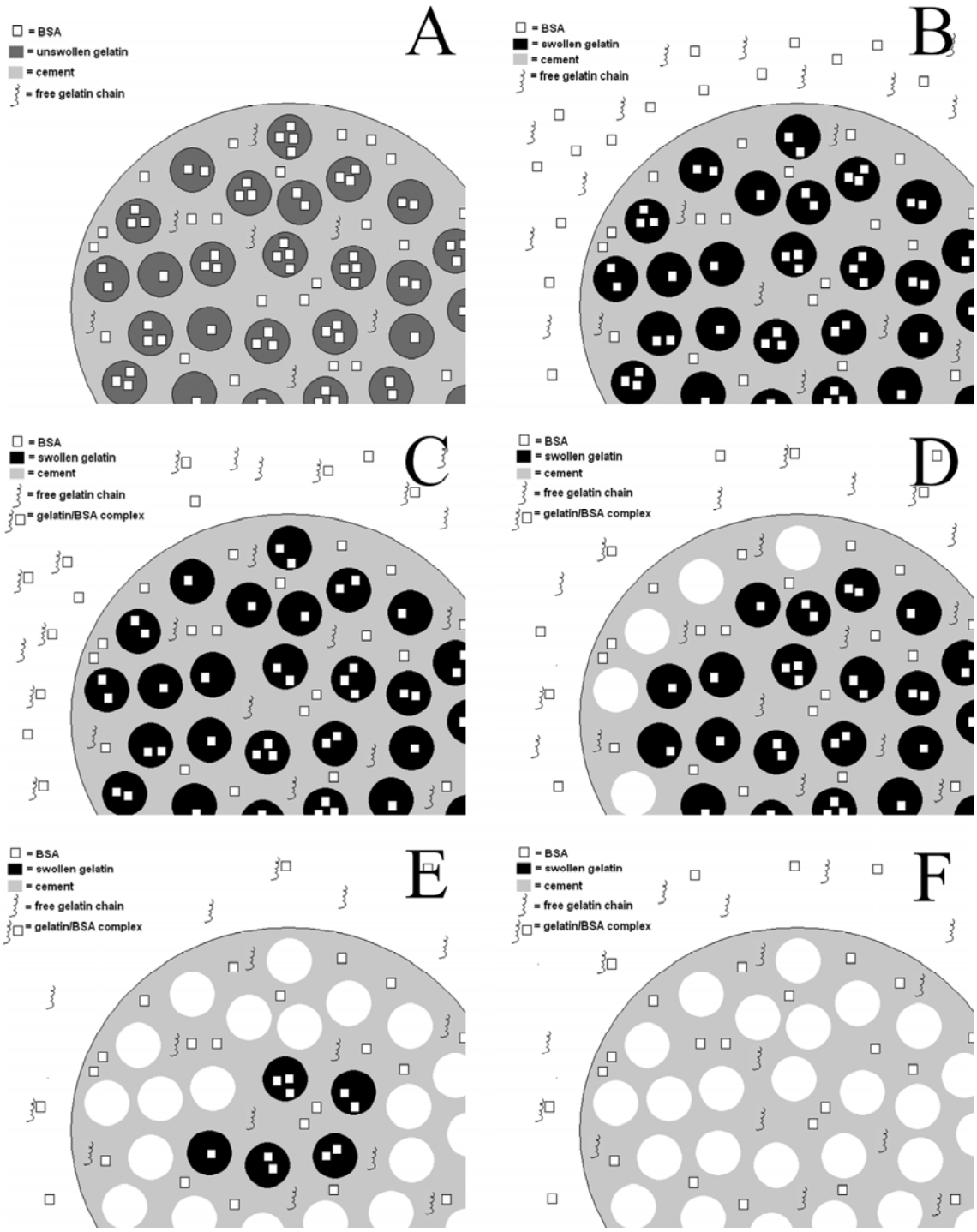



**Figure 10.** Representation of BSA release mechanism from GELA CPC; A= before release medium, B/C = delayed initial release of BSA from instant/prolonged loaded scaffolds, D/E = release during gelatin microsphere degradation, F = release from cement after microsphere degradation.

## 5. Conclusion

A 10% gelatin type A microsphere CPC was formulated with sufficient cement setting and cohesion properties, a gelatin microsphere content (macroporosity) of 58% and a compression strength of 18.5 MPa. *In vitro* degradation of the GELA CPC followed a surface-like erosion mechanism, in which a mass loss of 5% was obtained and the compression strength and E-modulus significantly decreased after 84 days in proteolytic medium. *In vitro* BSA release was tailored by loading gelatin microspheres

with BSA for a short (instant) or longer (prolonged) period before mixing them with the cement. Results showed a BSA release of more than 40% within 21 days with the instant loaded scaffolds, while prolonged loaded scaffolds showed a slower but sustained release. Differences were caused by weaker electrostatic interactions between the gelatin spheres and the BSA with the instant loaded scaffolds. SDS-PAGE also revealed differences in BSA structure between both loading mechanisms. Overall, 10wt% GELA microspheres can be used to generate *in situ* macroporosity into an injectable calcium phosphate cement and tailor release properties from these gelatin microsphere CPC composites.

*Acknowledgements*

Authors like to thank J.E. van den Dolder, V.M.J.I. Cuijpers and W.A. Loesberg for technical assistance. Scanning electron microscopy and confocal laser scanning microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) F.K. Kasper, T. Kushibiki, Y. Kimura, A.G. Mikos, Y. Tabata, In vivo release of plasmid DNA from composites of oligo(poly(ethylene glycol)fumarate) and cationized gelatin microspheres, J Control Release. 107 (2005) 547-561

2) A. Inoue, K.A. Takahashi, T. Arai, H. Tonomura, K. Sakao, M. Saito, M. Fujioka, H. Fujiwara, Y. Tabata, T. Kubo, The therapeutic effects of basic fibroblast growth factor contained in gelatin hydrogel microspheres on experimental osteoarthritis in the rabbit knee, Arthritis Rheum. 54(1) (2006) 264-270

3) H. Park, J.S. Temenoff, T.A. Holland, Y. Tabata, A.G. Mikos, Delivery of TGF-beta1 and chrondrocytes via injectable, biodegradable hydrogels for cartilage tissue engineering applications, Biomaterials. 26 (2005) 7095-7103

4) K.W. Wissemann, B.S. Jacobson, Pure gelatin microcarriers: synthesis and use in cell attachment and growth of fibroblast and endothelial cells, In Vitro Cell Dev Biol. 21(7) (1985) 391-401

5) Y. Tabata, Y. Ikada, Synthesis of gelatin microspheres containing interferon, Pharm Res. 6(5) (1989) 422-427

6) A. Muvaffak, I. Gurhan, N. Hasirci, Prolonged cytotoxic effect of colchicines release from biodegradable microspheres, J Biomed Mater Res Part B: Appl Biomater 71 (2004) 295-304

7) H-C. Liang, W-H. Chang, K-J. Lin, H-W. Sung, Genipin-crosslinked gelatin microspheres as a drug carrier for intramuscular administration: in vitro and in vivo studies, J Biomed Mater Res Part A. 65 (2003) 271-282

8) M. Yamamoto, Y. Takahashi, Y. Tabata, Controlled release by biodegradable hydrogels enhances the ectopic bone formation of bone morphogenic protein, Biomaterials. 24 (2003) 4375-4383

9) T.A. Holland, J.K.V. Tessmar, Y. Tabata, A.G. Mikos, Transforming growth factor-beta 1 release from oligo(poly(ethylene glycol) fumarate) hydrogels in conditions that model the cartilage wound healing environment, J Control Release. 94 (2004) 101-114

10) A.A. Ignatius, O. Betz, P. Augat, L.E. Claes, In vivo investigation on composites made of resorbable ceramics and poly(lactide) used as bone graft substitutes, J Biomed Mater Res Part A. 58(6) (2001) 701-709

11) W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen, Introduction of gelatin microspheres into an injectable calcium phosphate cement, J Biomed Mater Res Part A, published online January 11, 2008

12) C.J. Whitters, R. Strang, D. Brown, R.L. Clarke, R.V. Curtis, P.V. Hatton, A.J. Ireland, C.H. Lloyd, J.F. McCabe, J.W. Nicholson, S.N. Scrimgeour, J.C. Setcos, K. Sheriff, R. van Noort, D.C. Watts, D. Wood, Dental materials: 1997 literature review, J Dent. 27 (1999) 401-435

13) H.S. Katti, Biomaterials in total joint replacement, Colloid Surf B Biointerfaces. 39 (2004) 133-142

14) H.B. Gladstone, H.W. McDermott, D.D. Cooke, Implants for cranioplasty, Otolaryngol Clin North Am. 28(2) (1995) 381-400

15) E.M. Ooms, J.G.C. Wolke, M.T. van de Heuvel, B. Jeschke, J.A. Jansen, Histological evaluation of the bone response to calcium phosphate cement implanted in cortical bone, Biomaterials. 24(6) (2003) 989-1000

16) H.T. Peng, L. Martineau, P.N. Shek, Hydrogel-elastomer composite biomaterials: 3. Effects of gelatin molecular weight and type on the preparation and physical properties of interpenetrating polymer networks, J Mater Sci Mater Med. Published online july 31, 2007

17) V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials. 26 (2005) 5474-5491

18) H.P Stallmann, C. Faber, E.T. Slotema, D.M. Lyaruu, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11(hLF1-11) from calcium phosphate bone substitutes, J Antimicrob Chemother. 52 (2003) 853-855

19) H.P. Stallmann, C. Faber, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, In vitro gentamycin release from commercially available calcium-phosphate bone substitutes influence of carrier type on duration of the release profile, BMC Musculosketal Disorders. 7(18) (2006) 1-8

20) M. Yamamoto, Y. Ikada, Y. Tabata, Controlled release of growth factors based on biodegradation of gelatin hydrogel, J Biomater Sci Polym Edn. 12(1) (2001) 77-88

21) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics, J Biomater Sci Polym Edn. 17(9) (2006) 1057-1074

22) E. Van den Bosch , C. Gielens, Gel Chromatograpic fractionation and partial physicochemical characterization of gelatins, Chromatographia 58(7-8) (2003) 507-511

23) I. Khairoun , M.G. Boltong, F.C.M. Driessens, J.A. Planell, Effect of Calcium Carbonate on Clinical Compliance of Apatitic Calcium Phosphate Bone Cement, J Biomed Mater Res Part B: Appl Biomater. 38 (1997) 356-360

24) G. Carotenuto, G. Spagnuolo, L. Ambrosio, L. Nicolais, Macroporous hydroxyapatite as alloplastic materials for dental applications, J Mater Sci Mater Med. 10(10) (1999) 671-676

25) O. Gauthier, J.M. Bouler, E. Aguado, P. Pilet , G. Daculsi, Macroporous biphasic calcium phosphate ceramics: influence of macropore diameter and macroporosity percentage on bone ingrowth, Biomaterials. 19(1-3) (1998) 133-139

26) G. Daculsi, N. Passuti, Effect of the macroporosity for osseous substitution of calcium phosphate ceramics, Biomaterials. 11 (1990) 86-87

27) M-P. Ginebra, J-A. Delgado, L. Harr, A. Almirall, S. Del Valle, J.A. Planell, Factors affecting the structure and properties of an injectable self-setting calcium phosphate foam, J Biomed Mater Res Part A. 80 (2007) 351-361

28) W.J.E.M. Habraken, J.G.C. Wolke, J.A. Jansen, Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering, Adv Drug Deliv Rev. 59(4-5) (2007) 234-248

29) A. Gopferich, Mechanisms of polymer degradation and erosion, Biomaterials. 17 (1996) 103-114

30) E.A. Kremer, Y. Chen, K. Suzuki, H. Nagase, J.P. Gorski, Hydroxyapatite induces autolytic degradation and inactivation of matrix metalloprotease-1 and –3, J Bone Miner Res. 13(12) (1998) 1890-1902

31) A. Bigi, E. Boanini, S. Panzavolta, N. Roveri, K. Rubini, Bonelike apatite growth on hydroxyapatite-gelatin sponges from simulated body fluid, J Biomed Mater Res Part A. 59(4) (2001) 709-715

32) D.P. Link, J. van den Dolder, W.J.F.M. Jurgens, J.G.C. Wolke, J.A. Jansen, Mechanical evaluation of implanted calcium phosphate cement incorporated with PLGA microparticles, Biomaterials. 27 (2006) 4941-4947

33) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, Porous poly(DL-lactic-*co*-glycolic acid)/calcium phosphate cement composite for reconstruction of bone defects, Tissue Engineering. 12(4) (2006) 789-800

34) T.A. Holland, A.G. Mikos AG, Advances in drug delivery for articular cartilage, J Control Release. 86 (2003) 1-14

35) C. Perez, P. De Jesus, K. Griebenow, Preservation of lysozyme structure and function upon encapsulation and release from poly(lactic-co-glycolic) acid microspheres prepared by the water-in-oil-in-water method, Int J Pharm. 248 (2001) 193-206

36) E.J. Blom, J. Klein-Nulend, J.G.C. Wolke, K. Kurashina, M.A.J. van Waas, E.H. Burger EH, Transforming growth factor-β1 incorporation in an α-tricalcium phosphate monoxide cement: release characteristics and physicochemical properties, Biomaterials 23 (2002) 1261-1268

37) H. Seeherman , J.M. Wozney, Delivery of bone morphogenic proteins for orthopedic tissue regeneration, Cytokine Growth Factor Rev. 16 (2005) 329-345

38) P.Q. Ruhé , E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos AG, RhBMP-2 release from injectable poly(DL-lactic-*co*-glycolic acid)/calcium phosphate cement composites, J Bone Joint Surg Am. 85A(3) (2003) 75-81

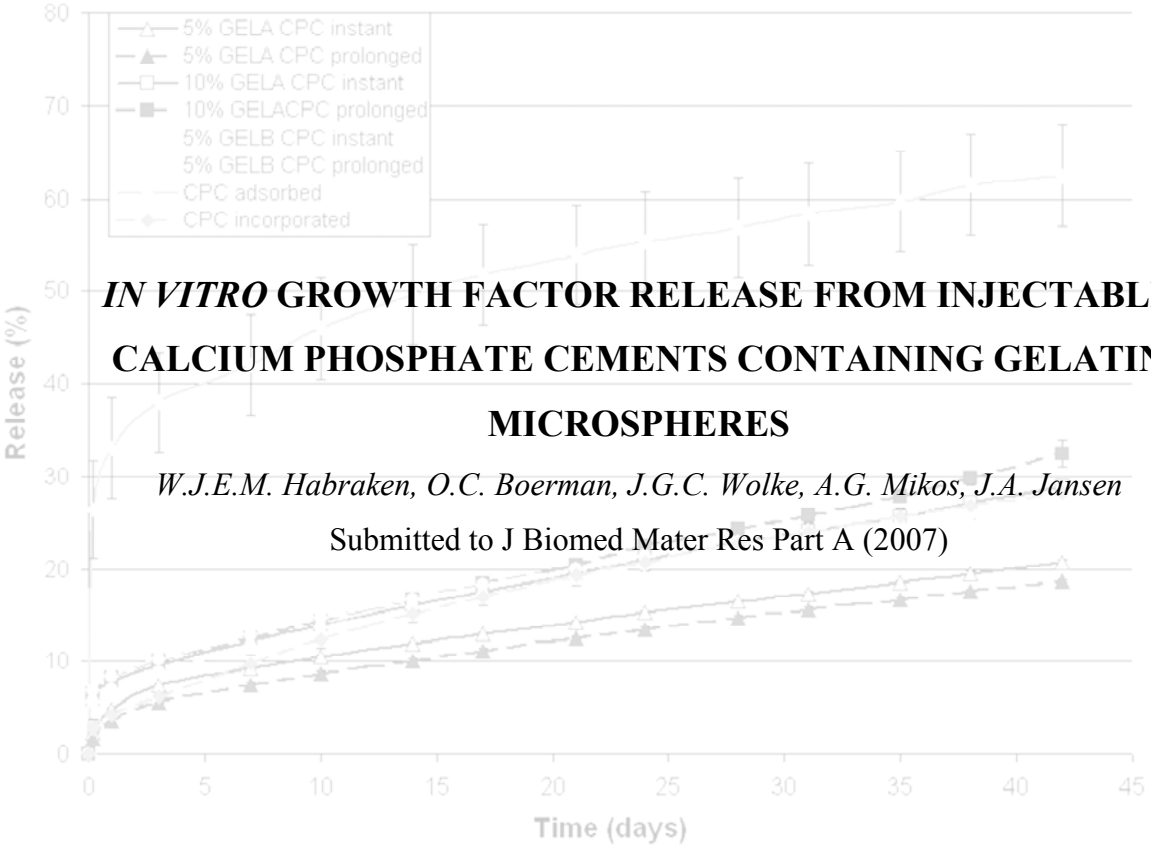
39) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, PLGA microsphere/calcium phosphate cement composites for tissue engineering: *in vitro* release and degradation characteristics, J Biomat Sci Polym Ed. Accepted for publication December 11, 2007

40) J. Ziegler, U. Mayr-Wohlfahrt, S. Kessler, D. Breitig, K-P Günther, Adsorption and release properties of growth factors from biodegradable implants, J Biomed Mater Res 59 (2002) 422-428

41) D.T. Hughes Wassel, R.C. Hall, G. Embery, Adsorption of bovine serum albumin onto hydroxyapatite, Biomaterials 16 (1995) 697-702

42) P.Q. Ruhé, O.C. Boerman, F.G.M. Russel, A.G. Mikos, P.H.M. Spauwen, J.A. Jansen, *In vivo* Release of rhBMP-2 Loaded Porous Calcium Phosphate Cement Pretreated with Albumin, J Mater Sci Mater Med. 17(10) (2006) 919-927

# CHAPTER 6




## *IN VITRO* GROWTH FACTOR RELEASE FROM INJECTABLE CALCIUM PHOSPHATE CEMENTS CONTAINING GELATIN MICROSPHERES

*W.J.E.M. Habraken, O.C. Boerman, J.G.C. Wolke, A.G. Mikos, J.A. Jansen*

Submitted to J Biomed Mater Res Part A (2007)

## 1. Introduction

Calcium phosphate cements (CPCs) are commonly used as bone-filling materials in the field of dentistry, orthopedic and post-operative surgery. These cements have proven be biocompatible and osteoconductive and due to the in-situ setting abilities, a perfect fit with the site defect can be accomplished[1-2]. One disadvantage is the high density and slow degradability of the material, which makes it less applicable for tissue engineering purposes[3]. Therefore, in earlier studies by our group microspheres made of poly(lactic-*co*-glycolic acid) (PLGA)[4-8] and gelatin[9-10] were added to an apatite cement. These microsphere CPCs exhibited good injectability characteristics and setting properties. During *in vitro/in vivo* degradation of the spheres, porosity increased concomitantly, yielding a structure of spherical pores and a decrease in mechanical strength. PLGA microspheres produced an acidic environment due to the hydrolysis of the ester groups[8]. On the other hand, gelatin microspheres degraded gradually by proteolysis and no additional decrease in pH was observed[9]. Drug release studies with microsphere CPC were also performed as the addition of osteoinductive drugs can improve bone remodeling at the implant site[11-12]. The drug was incorporated or adsorbed onto the microspheres to give a release pattern that differs from the usual diffusion dependent release from calcium phosphate cements[13-14]. *In vitro/in vivo* release data of bone morphogenic protein-2 (BMP-2) from PLGA microsphere CPC overall showed a pattern that was characterized by a small burst release, followed by a period of slow, sustained release[4-5]. Furthermore, by changing the molecular weight of the polymer release was tailored within certain ranges as low molecular weight PLGA microsphere CPC showed a significant higher release after 4 weeks *in vivo*[5]. In an *in vitro* release study using gelatin microsphere CPC[10], microspheres were loaded with bovine serum albumin (BSA) and release from the microsphere CPC was monitored for 9 weeks. Two loading methods were applied; BSA was loaded onto the microspheres directly before mixing (instant loading) or 24 h before mixing (prolonged loading) to establish a strong electrostatic bond between the basic gelatin (type A, pI = 7.0-9.0) and the acidic BSA (pI = 5.0)[15]. Results showed a release pattern without initial burst and a delayed release after 3 days that decreased in time. Release up to three weeks was significantly higher with the instant loaded scaffolds, whereas the prolonged loaded scaffolds exhibited a more sustained release. Structure analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed peaks of intact BSA after 3, 7 and 17 days. Gelatin microsphere CPC therefore seems to be a suitable scaffold for drug delivery, however, growth factors like BMP-2, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) can show a completely different release pattern than BSA[1]. In addition, there exist two types of gelatin; type A (pI = 7.0-9.0) and type B (pI = 4.5-5.0) that due to their opposite isoelectric point exhibit different electrostatic interactions with various drugs/proteins[15].

The goal of this study was to determine the release characteristics of gelatin type A or type B microsphere CPC by loading the scaffolds with various types of growth factor (TGF-β, bFGF and BMP-2) and applying two loading mechanisms. Also, different amounts of growth factor were loaded

onto the gelatin microspheres to investigate whether release from these composites is concentration dependent. Release was measured by labeling the growth factors with $^{125}I$ after which radioactivity of the loaded composites and release medium (phosphate buffered saline, PBS) was monitored for a total of 6 weeks.

## 2. Materials and Methods

### *2.1. Materials*

Cell culture tested gelatin type A (GELA) (pI = 7.0-9.0, bloom = 300) and gelatin type B (GELB) (pI = 4.7-5.2, bloom = 225) (Sigma-Aldrich, St. Louis, USA) were used for the fabrication of the microspheres. The calcium phosphate cement used is commercially available under the product name Calcibon® (Biomet Merck, Darmstadt, Germany) and consists of 61% α-tricalcium phosphate (α–TCP), 26% $Ca_2HPO_4$, 10% $CaCO_3$ and 3% precipitated hydroxyapatite (pHA). Collagenase 1A (Sigma) was used as gelatin degrading enzyme. Recombinant human transforming growth factor β1 (TGF-β1, pI = 9.5), basic fibroblast growth factor (bFGF, pI = 9.6) and bone morphogenic protein-2 (BMP-2, pI = 8.5) (R&D systems Europe Ltd., Abingdon, UK) were the growth factors used in the release experiment.

### *2.2. Methods*

#### *2.2.1. Preparation of gelatin microspheres*

2.5 g of GELA or GELB was dissolved in 25 ml dd$H_2O$ at an elevated temperature (30 min at 60°C). The resulting clear solution was added slowly (10 ml pipette) to a 250 ml three-necked round bottom flask containing 125 ml olive oil while stirring at 500 rpm (Teflon upper stirrer). During stirring, the round bottom flask was put in an ice bath. After 30 min, 50 ml of chilled acetone (4°C) and glutaraldehyde (0.5 ml = 6.25 mM) was added slowly. The solution was stirred for another hour. Microspheres were collected by filtration (D3, Schott Duran, Mainz, Germany) and washed several times with acetone (approx. 1 l) to remove residual olive oil. Following this, microspheres were stored in a drying chamber until further use. Morphology of the microspheres was visualized by scanning electron microscopy (SEM) (JEOL 6400-LINK AN 10000 at 10 kV) and is shown in Figure 1. Average size of the microspheres is given in Table 1 and was determined using digital image software (Leica Qwin®, Leica Microsystems AG, Wetzlar, Germany).

**Table 1.** Average size gelatin microspheres (n=200)

| | Average size (μm) | |
|---|---|---|
| | dry | swollen |
| GELA microspheres | 15.5 ± 13.8 | 37.4 ± 31.1 |
| GELB microspheres | 8.4 ± 7.6 | 20.7 ± 14.6 |

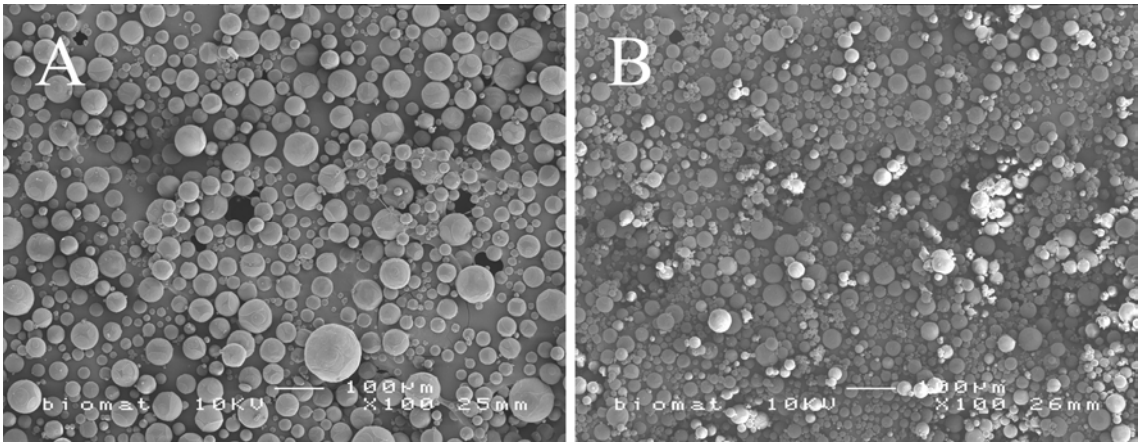



**Figure 1.** Morphology microspheres by scanning electron microscopy; A = GELA microspheres, B = GELB microspheres (original magnification 100x)

*2.2.2. Radioiodination of growth factors*

TGF-β1, bFGF and BMP-2 were labeled with $^{125}I$ according to the iodogen method[16]. Briefly, in a 500 µl eppendorf tube coated with 50 µg iodogen 10 µl of 0.5 M phosphate buffer saline (PBS) was added and adjusted to 100 µl with 50 mM PBS. Growth factor (8 µg) and 10-15 MBq $^{125}I$ (Perkin-Elmer, Boston, MA) was added and incubated at room temperature for 10 min. After that, 100 µl of saturated Tyrosine solution in PBS was added and labeling efficiency of the reaction was 36%, 13% and 43% for TGF-β1, bFGF and BMP-2 respectively. To remove the nonbound $I^{125}$ activity, the reaction mixture was eluted with 0.1% BSA in PBS on a prerinsed disposable Sephadex G25M column (PD-10; Pharmacia, Uppsala, Sweden). The radiochemical purity of the $^{125}I$-labeled growth factors was determined by instant thin layer chromatography (ITLC) on Gelman ITLC-SG strips (Gelman Laboratories, Ann Arbor, MI, USA) with 0.1M citrate, pH 5.0 as a mobile phase. The radiochemical purity of the growth factors is presented in Table 2.

**Table 2.** Radiochemical purity $^{125}I$-labeled growth factors

| | TGF-β1 | bFGF | BMP-2 |
|---|---|---|---|
| Radiochemical purity | 95.6% | 83.9% | 97.8% |

*2.2.3. Preparation of gelatin microsphere/calcium phosphate cement composites*

For the release study, 10wt% gelatin type A microsphere CPCs (GELA CPCs) and 5wt% gelatin type B microsphere CPCs (GELB CPCs) were prepared as described in previous studies[9-10]. The weight% of microspheres inside the cement corresponds to the maximum amount of microspheres that can be incorporated to still obtain a composite with manageable handling properties[10]. TGF-β1, bFGF and BMP-2 was loaded onto the composites by two loading methods where growth factor was mixed with the cement directly after loading them to the microspheres (instant loading), or following a period of swelling before cement was added (prolonged loading). Briefly, growth factor loaded 5/10 wt% gelatin microsphere CPCs were made by adding a growth factor solution in PBS (300/560 µl) to 50/100 mg of

dry gelatin microspheres inside a BSA coated 2 ml syringe. With instant loading, after 15 s vigorously stirring using a Silamat® (Vivadent, Schaan, LIE) mixing apparatus, 950/900 mg calcium phosphate cement was added and the constituents were stirred again for 15 s or until an equal distribution of spheres inside the cement was obtained. With the prolonged loaded composites, the gelatin microspheres were allowed to swell for 24 h at 4°C before they were mixed with the cement. Following both procedures, 280 µl of 1% $Na_2HPO_4$ was added and after 15s of stirring, the resulting paste was injected into Teflon moulds (cylindrical, d = 4.5 mm, h = 9.0 mm). Samples were hardened at room temperature overnight. SEM-micrographs of the gelatin microsphere CPCs are given in Figure 2. Growth factor-loaded samples without microspheres (CPC) were used as control and prepared by adding a growth factor solution to preset cement samples (adsorbed). Calculated amount of growth factor per sample is 50 ng. Furthermore, drug entrapment efficiency was determined by measuring the γ-irradiation of the preset samples after which it was divided by the γ-irradiation of the corresponding calculated amount of labeled growth factor.

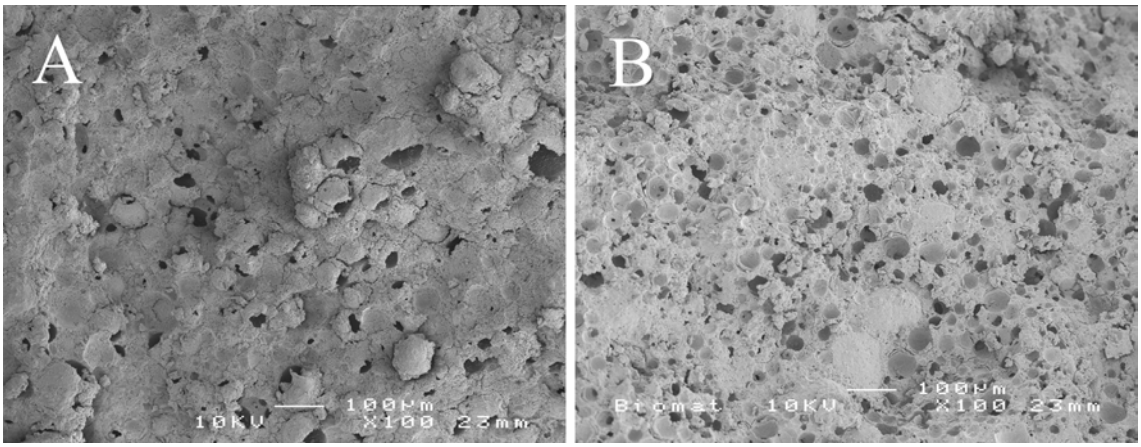



**Figure 2.** Morphology gelatin microsphere CPCs; A = 10% GELA CPC, B = 5% GELB CPC (original magnification 100x)

**Table 3.** Vol% of microspheres inside gelatin microsphere CPC

| Formulation | Volume % of microspheres |
|---|---|
| 5% GELA CPC | 48.7 ± 1.8 % |
| 10% GELA CPC | 57.5 ± 1.4 % |
| 5% GELB CPC | 48.4 ± 1.8 % |

With BMP-2 some supplementary studies were performed. To investigate whether growth factor release is concentration dependent, different amounts of BMP-2 were incorporated into the cement. Next to 50 ng of $^{125}$I-labbeled BMP-2 per scaffold, samples were loaded with 500 ng and 5 µg of BMP-2 applying hot/cold ratios of 1/10 and 1/100. Furthermore to investigate the effect on drug release of the total amount/volume of incorporated microspheres, with BMP-2 also a 5wt% GELA CPC was formulated. Volume% of gelatin microspheres inside the 5 and 10wt% GELA CPC[10] and 5wt% GELB CPC is presented in Table 3. Finally, in addition to the adsorbed CPC controls, CPC

samples were prepared where BMP-2 was dissolved into the liquid hardener (1% $Na_2HPO_4$) during cement preparation (incorporated).

*2.2.4. Release study*

Samples were placed in 3 ml PBS containing 373 ng/ml collagenase 1A[17] inside 10 ml polypropylene tubes. Tubes were put in a water bath containing a rotating plate (70 rpm) at 37°C. Medium was renewed every 3-4 days. At the same time, γ-irradiation of the sample/medium was measured for a total of 6 weeks. Samples were taken and measured in triplicate (n = 3).

*2.2.5. Statistical analysis*

Data were presented as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat®.

## 3. Results

The drug entrapment efficiencies of the samples are given in Table 4. For all types of gelatin microsphere CPC, drug entrapment efficiencies were around 70-75%. Differences in entrapment efficiency between instant and prolonged loaded scaffolds were observed, though no clear trend was observed. CPC controls where the BSA was adsorbed exhibited higher entrapment efficiencies (82-94%) than the gelatin microsphere CPC, though the incorporated CPC showed an efficiency of only 51%.

**Table 4.** Entrapment efficiency gelatin microsphere CPC and CPC as percentage of calculated amount of growth factor (1x = 50 ng, 10x = 500 ng, 100x = 5 μg)

| | TGF-β1 | bFGF | BMP-2 | | |
|---|---|---|---|---|---|
| | | | 1x | 10x | 100x |
| CPC (adsorbed) | 81.7 ± 3.4 | 82.3 ± 3.1 | 93.7 ± 4.2 | 90.7 ± 4.7 | 88.5 ± 2.9 |
| 10% GelA CPC instant | 74.8 ± 1.8 | 75.7 ± 2.6 | 77.7 ± 2.3 | 78.8 ± 1.5 | 75.7 ± 1.9 |
| 10% GelA CPC prolonged | 66.8 ± 1.7 | 76.1 ± 0.5 | 71.0 ± 2.0 | 72.1 ± 1.3 | 68.3 ± 1.6 |
| 5% GelB CPC instant | 72.4 ± 2.8 | 69.4 ± 0.4 | 77.8 ± 2.4 | 81.0 ± 4.3 | 73.1 ± 4.4 |
| 5% GelB CPC prolonged | 68.5 ± 0.2 | 68.8 ± 1.6 | 71.4 ± 0.9 | 71.1 ± 1.0 | 70.0 ± 1.3 |
| CPC (incorporated) | - | - | 50.9 ± 2.3 | - | - |
| 5% GelA CPC instant | - | - | 69.8 ± 1.5 | - | - |
| 5% GelA CPC prolonged | - | - | 75.8 ± 1.8 | - | - |

The release pattern for the CPC is given in Figure 3. Overall, CPC where the drug was adsorbed onto the surface exhibited a release pattern consisting of an initial burst, followed by a sustained release that was slowly diminishing in time. The release pattern of both BMP-2 and bFGF was similar, whereas the TGF-β1 showed a significant lower initial burst and sustained release. In contrast to the BMP-2 adsorbed CPC, release from the BMP-2 incorporated cement did not show an initial burst.

Furthermore, sustained release was comparable to the BMP-2 adsorbed cement though release efficiency (Table 5), due to the initial burst, was significantly lower ($p<0.001$).

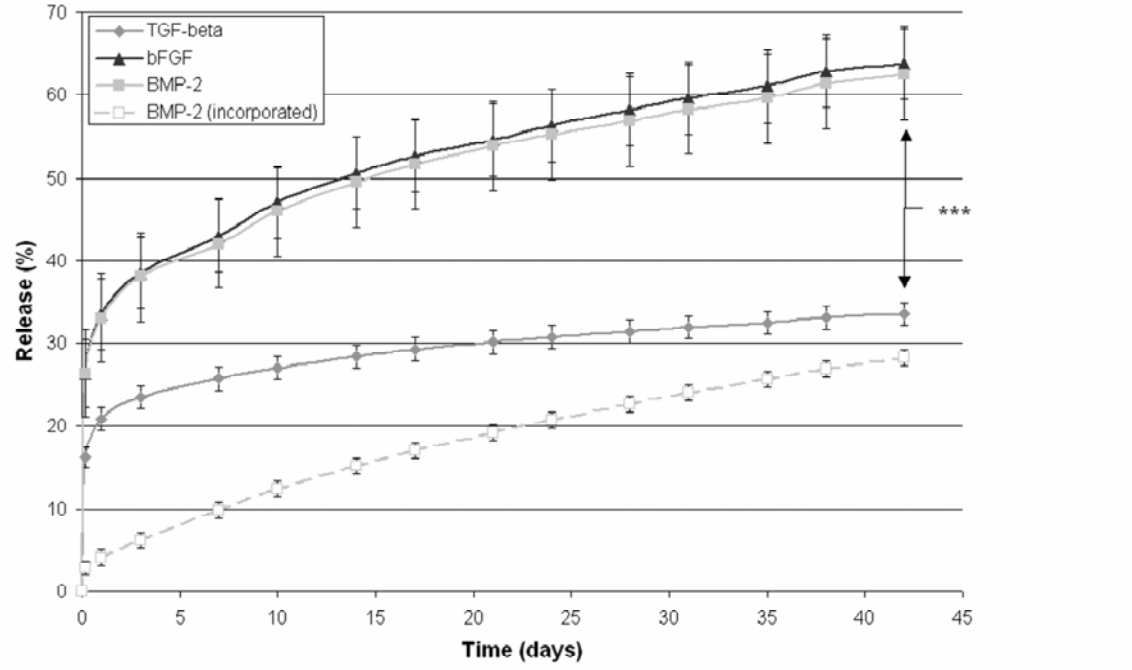



**Figure 3.** Growth factor release from CPC, *** = $p < 0.001$

**Table 5.** Release efficiency after 6 weeks of gelatin microsphere CPC and CPC as percentage of actual loaded protein (1x = 50 ng, 10x = 500 ng, 100x = 5 µg)

| | TGF-β1 | bFGF | BMP-2 | | |
|---|---|---|---|---|---|
| | | | 1x | 10x | 100x |
| CPC (adsorbed) | 33.5 ± 1.4 | 63.9 ± 4.4 | 62.5 ± 5.4 | 56.2 ± 2.8 | 57.4 ± 2.3 |
| 10% GelA CPC instant | 13.3 ± 0.3 | 50.5 ± 1.2 | 28.8 ± 0.3 | 30.5 ± 1.0 | 29.2 ± 0.5 |
| 10% GelA CPC prolonged | 17.8 ± 0.5 | 54.3 ± 1.4 | 32.5 ± 1.4 | 32.0 ± 0.8 | 34.2 ± 1.4 |
| 5% GelB CPC instant | 13.8 ± 0.2 | 53.7 ± 0.4 | 27.1 ± 0.3 | 28.0 ± 1.0 | 28.3 ± 0.6 |
| 5% GelB CPC prolonged | 14.4 ± 0.4 | 50.7 ± 1.0 | 28.1 ± 0.4 | 28.0 ± 0.6 | 28.3 ± 0.2 |
| CPC (incorporated) | - | - | 28.3 ± 1.0 | - | - |
| 5% GelA CPC instant | - | - | 20.7 ± 0.3 | - | - |
| 5% GelA CPC prolonged | - | - | 18.6 ± 0.3 | - | - |

Similar to the CPC samples, both 10% GELA CPCs (Figure 4) and 5% GELB CPCs (Figure 5) exhibited a pattern consisting of a (small) initial burst followed by sustained release of the growth factor. Also here, release was dependent on the type of growth factor, where bFGF exhibited a higher burst release than BMP-2 or TGF-β1. Sustained release and release efficiency (Table 5) was in the following order; bFGF > BMP-2 >> TGF-β1. The 10% GELA CPC also showed a significant higher sustained release from the prolonged loaded scaffolds when compared to the instant loaded scaffolds for all growth factors. With TGF-β1 the prolonged loaded scaffolds experienced a 33% higher release after 6 weeks, whereas with bFGF and BMP-2 this was 8 and 13%. The 5% GELB CPC did not show

such a trend, as with bFGF the instant loaded scaffolds showed a significant higher release ($p<0.01$), and differences between instant and prolonged loaded scaffolds with BMP-2 and TGF-β1 were small.

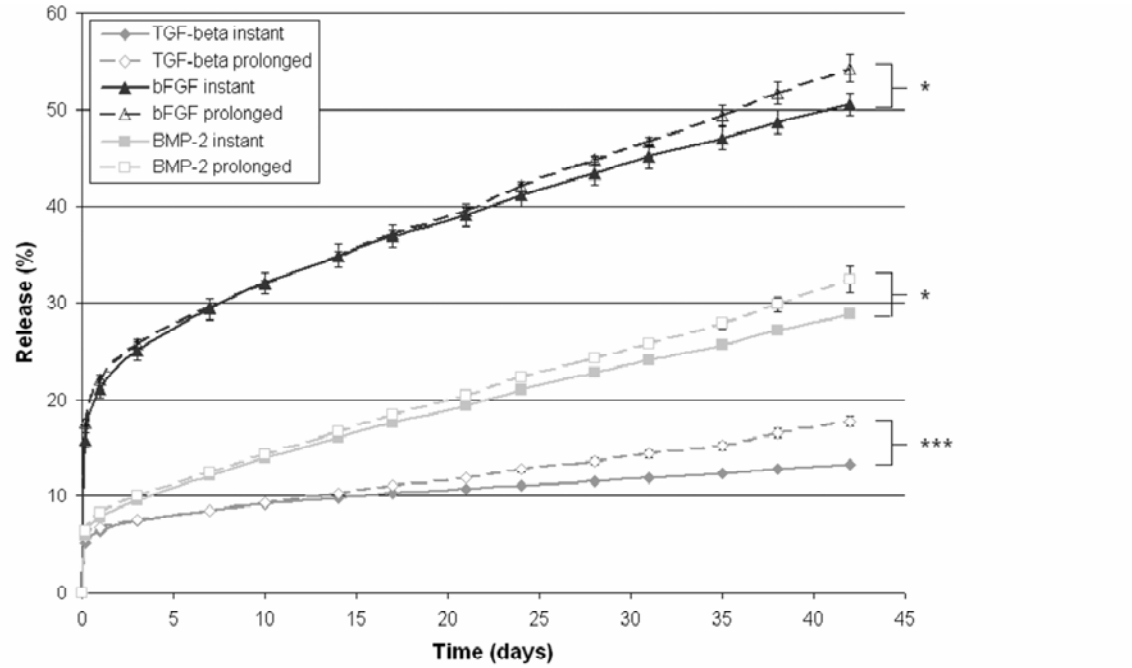



**Figure 4.** Growth factor release from 10% GELA CPC, * = $p < 0.05$, *** = $p < 0.001$

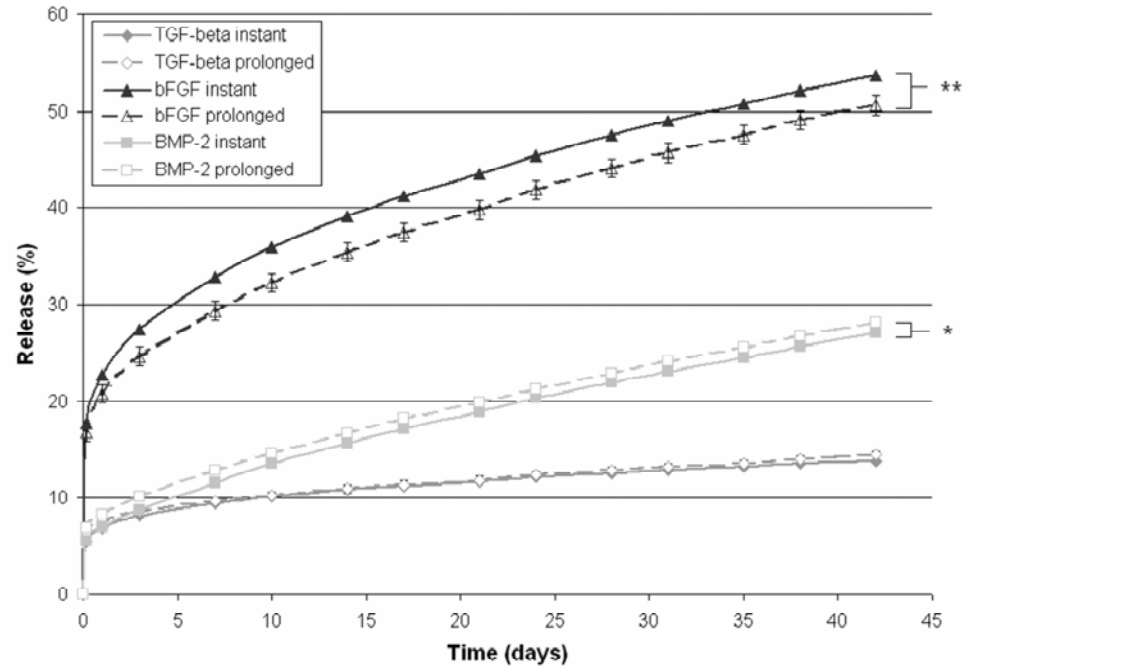



**Figure 5.** Growth factor release from 5% GELB CPC, * = $p < 0.05$, ** = $p < 0.01$

In Figure 6 the BMP-2 release from all formulations is depicted. It was observed that sustained release from the 5% GELA CPC was significantly lower ($p<0.001$) than the 10% GELA CPC, 5% GELB CPC and CPC controls. Furthermore, sustained release from the incorporated CPC was identical to the 5%

GELB CPC and instant loaded 10% GELA CPC, though slower than the prolonged loaded 10% GELA CPC.

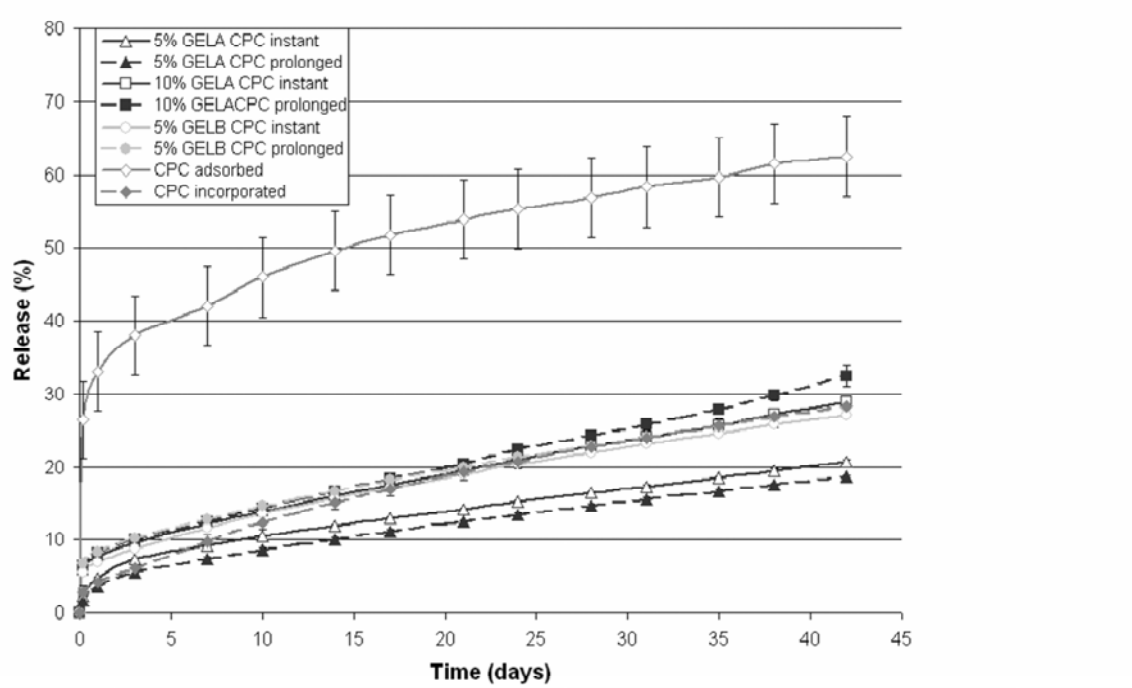



**Figure 6.** BMP-2 release from gelatin microsphere CPC and CPC

Figure 7 shows the absolute release from the BMP-2 loaded composites. The release patterns at the different BMP-2 concentrations were identical for all formulations and exhibited respectively a 10/100 fold increase in burst or sustained release and absolute release after 6 weeks (Table 5) when implants were loaded with a 10/100 fold higher amount of BMP-2.

## 4. Discussion

In the present study, growth factor release from injectable calcium phosphate cements with incorporated gelatin microspheres was investigated. For this purpose, TGF-β1, bFGF or BMP-2 were added to the gelatin microspheres before they were mixed with the cement using two loading methods. Furthermore, scaffolds were prepared using either type A or type B gelatin microspheres. In addition, for BMP-2 the effect of growth factor concentration and amount of incorporated microspheres on the release pattern was investigated. Samples without gelatin microspheres (CPC) were used as control where the growth factor was adsorbed onto the scaffolds or added to the liquid hardener (BMP-2).

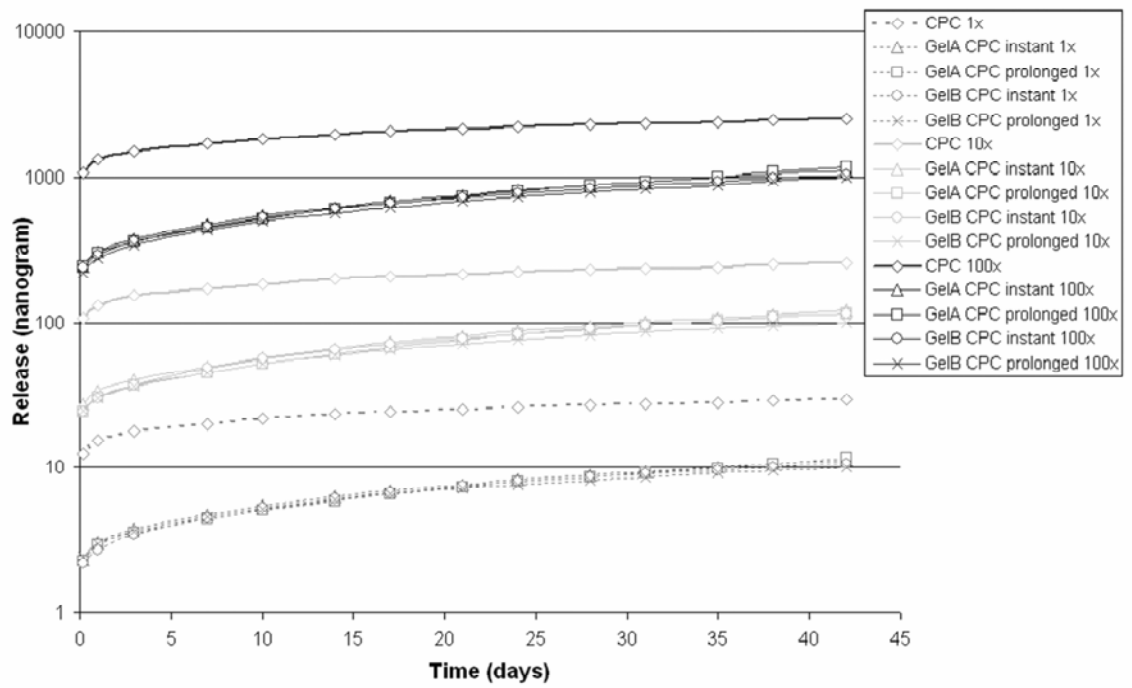



**Figure 7.** Cumulative BMP-2 release from 10% GELA CPC, 5% GELB CPC and CPC with different amount of incorporated BMP-2 (1x = 50 ng, 10x = 500 ng, 100x = 5 μg)

Results showed that the drug entrapment efficiency of the CPC controls was dependent on the loading method as the incorporated CPC sample clearly exhibited a significant lower efficiency than the adsorbed CPC samples. For the incorporated CPC sample, losses occurred when drug adsorbed to the syringe during mixing or when drug was absorbed to the Teflon mould during cement hardening. Entrapment efficiency of the gelatin microsphere composites was intermediate. This indicates that the electrostatic interaction between the drug and microspheres was strong enough to retain drug inside the spheres during mixing, however, part of the growth factor will be dispersed into the surrounding cement[15].

With most formulations an initial burst release of the growth factor was observed. With the growth factor-adsorbed CPC most of the drug was located at the outside of the sample, which explains the high burst release[13-14]. The CPC with incorporated BMP-2 did not show a burst release as the growth factor in this sample was dispersed more evenly through the cement, which is known for its protein-binding capacity[13-14, 18]. Occasionally, with the gelatin microsphere CPCs a substantial burst release was obtained. Whereas the BMP-2 and TGF-β1 loaded composites showed a burst release of 5-7%, with bFGF a burst release of 16-18% was observed. This burst release is probably due to the suboptimal radiochemical purity of $I^{125}$ labelled bFGF, which was 84%. The radioactive preparation used in this experiment therefore contained 16% free Iodine-125 that is expected to exhibit a fast release from the scaffolds. Regarding this radiochemical purity, burst release from gelatin microsphere CPC was low. As gelatin scaffolds/microspheres often show a high burst release[15, 17, 19], the low burst observed here is a result of protein binding to the cement after release from the microspheres.

Following the burst release, with each growth factor a sustained release was observed. With the CPC controls, this sustained release is probably due to diffusion of drug through the cement[20,21] as the CPC does not show signs of degradation or dissolution[8-10]. Figure 8 shows that BMP-2 release from the incorporated CPC is proportional to the square root of time, which is typical for a diffusion dependent release. Prolonged loaded 10% GELA CPC, however, shows a more linear sustained release. With the gelatin microsphere CPCs drug release can also be the result of the proteolytic degradation of microspheres. Supposedly degradation of gelatin microspheres inside the CPC is a gradual process from the outside of the implant to the inner part[10], though in these samples also an initial burst of free gelatin chains was observed. According to literature[15], electrostatically bonded growth factor releases concomitantly with release of gelatin degradation products. The observation that the release from the 10% GELA CPC is more rapid than from the 5% GELA CPC indicates that a similar mechanism indeed occurs. Furthermore as the used collagenase specifically degrades collagen or gelatin[22], no proteolytic degradation of the growth factor is expected.

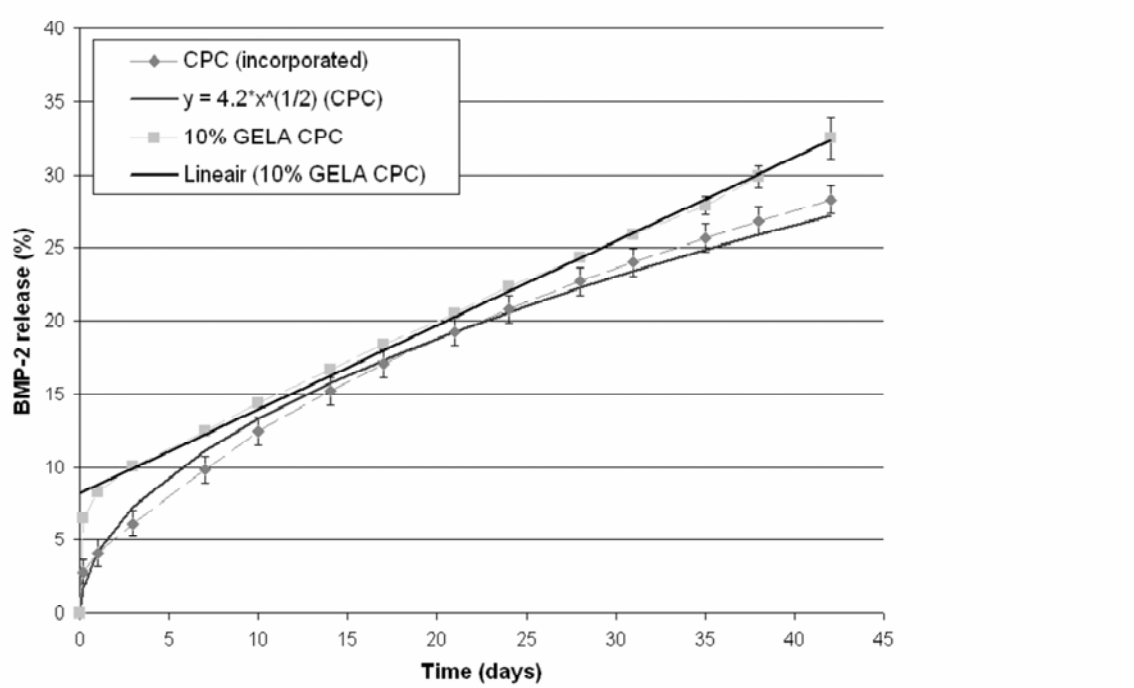



**Figure 8.** BMP-2 release curves from BMP-2 incorporated CPC and 10% GELA CPC with additional trend lines

In most cases the magnitude of the sustained release from the gelatin microsphere CPC was comparable to the CPC samples, whereas it was significantly lower with 5% GELA CPC. This can be due to the fact that proteolytic gelatin degradation does not occur in a high extent for the 5% GELA CPC and most of the growth factor is retained in the gelatin microspheres. In previous degradation studies[9-10] it was concluded that due to the low interconnectivity of the microspheres inside a 5% gelatin microsphere CPC, the enzyme did not penetrate the composite as it experienced physical interactions with the surrounding cement. The release that is observed from the 5% GELA CPC is mostly due to diffusion from the cement, which is higher with the CPC controls. Figure 6 also shows

that BMP-2 release from the 5% GELB CPC is significantly higher than release from the 5% GELA CPC, whereas the vol% of microspheres inside the composite is similar (Table 3). This can be explained by a better distribution of the GELB microspheres into the cement, which are smaller in size than the GELA microspheres, leading to a higher interconnectivity and improved microsphere degradation. Also, the lower isoelectric point of gelatin type B can have caused the higher sustained release from the GELB CPC, as the used growth factors all have an isoelectric point of 8.5-9.5. The electrostatic interactions with the acidic GELB microspheres (pI = 4.5-5.0) will be stronger than with the GELA microspheres (pI = 7.0-9.0), leading to smaller losses to the surrounding cement during composite preparation. Similarly, an increase in the % of microspheres inside the GELB CPC is supposed to improve drug release from these composites. However, like food-grade gelatin microspheres in a previous study[9] with GELB microspheres the addition of more than 5wt% of microspheres renders a composite with insufficient setting properties.

Apart from the implant material, the release test showed large differences in the sustained release of the various growth factors, i.e. showing a fast release of bFGF and a much slower release of TGF-β1. In agreement with other studies with cement or gelatin as scaffold material[15, 19, 23],release in this study was highly dependent on the type of growth factor. Intrinsic and physical parameters like the isoelectric point[15, 19], hydration[24] and size of the drug play an important role in drug release, however, these parameters not always have the same effect on the release pattern/efficiency. Charge distribution and chain conformation of the drug are markedly influenced by environmental conditions like buffer capacity (pH) and consistency of the medium[15, 24]. Also the state of the cement surface changes when these environmental conditions alter, resulting in a more positively/negatively charged substrate[25]. It is therefore not always straightforward to predict release from these calcium phosphate cements, despite the similarities between the drugs.

In the previous BSA release study[10] it was shown that the loading method also can be used to tailor release from the 10% GELA CPCs. In accordance to this study, current results indeed showed that absorption of the growth factor for a longer period onto the microspheres (prolonged loading), led to a higher sustained release with these composites. The explanation for this phenomenon is a higher release from the gelatin microspheres during proteolytic degradation as result of stronger electrostatic interactions between the gelatin and growth factor, and less losses to the surrounding cement. 5% GELA CPC and 5% GELB CPC do not show this feature, or to a lesser extent. As discussed earlier, proteolytic degradation did not occur in a high extent for the 5% GELA CPC and therefore it is not surprising that with this formulation the prolonged loaded samples did not show a higher sustained release than the instant loaded samples. Furthermore, as electrostatic interactions between the gelatin and growth factors are expected to be stronger with GELB microspheres, these interactions already could be optimal using instant loading.

Another feature that was observed in the BSA release study[10] was a substantial higher release from the instant loaded scaffolds within the first weeks due to weaker electrostatic interactions between the

drug and gelatin. In our study, in most cases growth factor release up to 3 weeks was similar for both instant and prolonged loaded samples. With bFGF loaded 5% GELB CPC there was a 10% higher release from the instant loaded scaffolds within the first weeks, though with BSA a 100% higher release was obtained. Comparison of both studies, the type of drug (BSA/growth factor), substrate medium (demineralized water/PBS) and drug concentration (1.5 mg/50-5000 ng) used during the release experiment all could have influenced drug release. The use of demineralized water in the BSA release study also resulted in a strong decrease of the pH. An increase in drug concentration can influence release properties as protein-binding sites are saturable[26]. Increasing BMP-2 loading from 50 ng to 5 μg in this study did not lead to a different release pattern or efficiency, but a proportional increase in absolute release. Within this range, sustained release from the gelatin microsphere CPCs and CPC controls therefore can be tuned to a certain therapeutic amount per week.

## 5. Conclusion

Gelatin microsphere / calcium phosphate cement composites can be applied for the sustained release of growth factors. The composites showed release efficiencies of 14-55% and release patterns consisting of a small initial burst followed by a sustained release up to 6 weeks. CPC controls showed a higher burst release as well as release efficiency when drug was adsorbed at the outside of the sample, but a comparable pattern and release efficiency when drug was incorporated during cement preparation. Sustained release from the gelatin microsphere CPCs was highly dependent on the type of growth factor but was also dependent on the total volume of microspheres and type of gelatin. Also the loading mechanism was important, as instant loaded 10% GELA CPC showed a significant higher sustained release than prolonged loaded composites. Furthermore, release patterns/efficiencies from the gelatin microsphere composites and CPC controls did not differ significantly when BMP-2 concentration was increased with a factor of 10 -100.

*Acknowledgements*

Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) W.J.E.M. Habraken, J.G.C. Wolke, J.A. Jansen, Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering, Adv Drug Deliv Rev. 59(4-5) (2007) 234-48

2) E.M. Ooms, J.G.C. Wolke, M.T. van de Heuvel, B. Jeschke, J.A. Jansen, Histological evaluation of the bone response to calcium phosphate cement implanted in cortical bone, Biomaterials. 24(6) (2003) 989-1000

3) L. Comuzzi, E.M. Ooms, J.A. Jansen, Injectable calcium phosphate bone cement as a filler for bone defects around oral implants: an experimental study in goats, Clin Oral Implants Res. 13(3) (2002) 304-11

4) P.Q. Ruhé, E.L. Hedberg, N.T. Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, rhBMP-2 release from injectable poly(DL-lactic-co-glycolic acid)/calcium-phosphate cement composites, J Bone Joint Surg Am. 85A(3) (2003) 75-81

5) P.Q. Ruhé, O.C. Boerman, F.G. Russel, P.H.M. Spauwen, A.G. Mikos, J.A. Jansen, Controlled release of rhBMP-2 loaded poly(dl-lactic-co-glycolic acid)calcium phosphate cement composites in vivo, J Control Release. 106(1-2) (2005) 162-71

6) P.Q. Ruhé, E.L. Hedberg, N.T. Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, Biocompatibility and degradation of poly(DL-lactic-co-glycolic acid)calcium phosphate cement composites, J Biomed Mater Res Part A 74(4) (2005) 533-544

7) P.Q. Ruhé, E.L. Hedberg-Dirk, N.T. Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos, Porous poly(DL-lactic-co-glycolic acid)/calcium phosphate cement composite for reconstruction of bone defects, Tissue Eng. 12(4) (2006) 789-800

8) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics, J Biomater Sci Polym Ed. 17(9) (2006) 1057-1074

9) W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen, Introduction of gelatin microspheres into an injectable calcium phosphate cement, J Biomed Mater Res Part A *in press*

10) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Porcine gelatin microsphere/calcium phosphate cement composites: *in vitro* degradation and drug release, *submitted to Journal of Biomedical Materials Research Part B: Applied Biomaterials*

11) H. Seeherman, J.M. Wozney, Delivery of bone morphogenic proteins for orthopedic tissue regeneration, Cytokine & Growth Factor Rev. 16 (2005) 329-345

12) J.A. Jansen, J.W.N. Vehof, P.Q. Ruhé, H. Kroeze-Deutman, Y. Kuboki, H. Takita, E.L Hedberg, A.G. Mikos, Growth factor-loaded scaffolds for bone engineering, J Control Release. 101 (2005) 127-35

13) H.P. Stallmann, C. Faber, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, In vitro gentamycin release from commercially available calcium-phosphate bone substitutes influence of carrier type on duration of the release profile, BMC Musculoskeletal Disorders. 7(18) (2006) 1-8

14) H.P. Stallmann, C. Faber, E.T. Slotema, D.M. Lyaruu, A.L.J.J. Bronckers, A.V. Nieuw Amerongen, P.I.J.M. Wuisman, Continuous-release or burst release of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11) from calcium phosphate bone substitutes, J Antimicrob Chemother. 52 (2003) 853-855

15) Yamamoto M, Ikada Y, Tabata Y. Controlled release of growth factors based on biodegradation of gelatin hydrogel. J Biomater Sci Polym Ed 2001;12(1):77-88

16) P.J. Fraker, L.C. Speck Jr., Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,9-tetrachloro-3a,6a-diphrenylglycoluril, Biochem Biophys Res Commun. 80 (1978) 849-857

17) T.A. Holland, J.K. Tessmar, Y. Tabata, A.G. Mikos, Transforming growth factor-beta 1 release from oligo(poly(ethylene glycol) fumarate) hydrogels in conditions that model the cartilage wound healing environment, J Control Release. 94(1) (2005) 101-114

18) E.J. Blom, J. Klein-Nulend, J.G.C. Wolke, K. Kurashina, M.A.J. van Waas, E.H. Burger, Transforming growth factor-β1 incorporation in an α-tricalcium phosphate/dicalcium phosphate dehydrate/tetracalcium phosphate monoxide cement: release characteristics and physicochemical properties, Biomaterials. 23 (2002) 1261-1268

19) T.A. Holland, Y. Tabata, A.G. Mikos, Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering, J Control Release. 101(1-3) (2005) 111-125

20) M.P. Ginebra, T. Traykova, J.A. Planell, Calcium phosphate cements as bone drug delivery systems: A review, J Control Release. 113 (2006) 102-110

21) N. Faisant N, J. Siepmann, J. Richard, J.P. Benoit, Mathematical modeling of drug release from bioerodible microparticles: effect of gamma-irradiation, Eur J Pharm Biopharm. 56 (2003) 271-279

22) T.A. Holland, A.G. Mikos, Advances in drug delivery for articular cartilage, J Control Release. 86 (2003) 1-14

23) J. Ziegler, U. Mayr-Wohlfart, S. Kessler, D. Breitig, K-P. Günther, Adsorption and release properties of growth factors from biodegradable implants, J Biomed Mater Res Part A. 59 (2002) 422-428

24) D.T. Hughes Wassell, R.C. Hall, G. Embery, Adsorption of bovine serum albumin onto hydroxyapatite, Biomaterials. 16 (1995) 697-702

25) L.S. Harding, H. Rashid, K.A. Hing, Surface charge and the effect of excess calcium ions on the hydroxyapatite surface, Biomaterials. 26 (2005) 6818-6828

26) P.Q. Ruhé, O.C. Boerman, F.G.M. Russel, A.G. Mikos, P.H.M. Spauwen, J.A. Jansen, *In vivo* Release of rhBMP-2 Loaded Porous Calcium Phosphate Cement Pretreated with Albumin, J Mater Sci Mater Med. 17(10) (2006) 919-927

# CHAPTER 7

## INTRODUCTION OF ENZYMATICALLY DEGRADABLE POLY(TRIMETHYLENE CARBONATE) MICROSPHERES INTO AN INJECTABLE CALCIUM PHOSPHATE CEMENT

*W.J.E.M. Habraken, Z. Zhang, J.G.C. Wolke, D.W. Grijpma, A.G. Mikos, J. Feijen, J.A. Jansen*

Biomaterials. 29(16) (2008) 2464-2476

## 1. Introduction

Calcium phosphate cements are used in clinical practice for filling up bone defects because of their biocompatibility, osteoconductivity and perfect fit with the surrounding tissue when injected *in situ*[1]. For bone tissue engineering purposes, resorption of dense cement is too slow[2]. Therefore, (macro)porosity is often introduced to enable bone ingrowth into the material concomitantly improving the bioresorption[3]. Incorporation of poly(lactic-*co*-glycolic) acid (PLGA) or gelatin microspheres in calcium phosphate cements[4-6] has proven to introduce macroporosity during *in situ* degradation of the microspheres without unacceptably affecting the handling properties or cement setting. A rationale for this construct is that bone ingrowth compensates for the loss of mechanical strength, which occurs as a result of microsphere degradation. Furthermore, these microspheres can be used as drug delivery vesicles for osteoinductive growth factors[7-8], thereby improving the often marginal release from these cements.

Despite the favorable release of growth factors we observed also that inclusion of PLGA microspheres was associated with a loss of mechanical strength of the set cement as well as the production of acidic degradation products[4]. The subsequent replacement of PLGA with gelatin microspheres resulted in a more gradual degradation, which occurred from the outer to the inner part of the cement composite, where degradation was dependent on the type and amount of incorporated microspheres[5-6]. Unfortunately, the gelatin degrading enzyme (collagenase) experienced physical interactions with the surrounding calcium phosphate cement, delaying degradation of microspheres at the inner parts. Another setback is that gelatin exhibits biomimetic properties[9]. As a consequence, the microspheres became often completely covered by a calcium phosphate precipitate that decreased the interconnectivity of the spheres and thereby also penetration of the enzyme.

Poly(trimethylene carbonate) (PTMC, Figure 1) is a flexible, biodegradable polymer that in contrast to PLA/PLGA does not produce acidic degradation products[10]. PTMC degrades via surface erosion, i.e. degradation occurs only at the surface resulting in a decrease of size/radius of the material[11]. Studies have shown that this process is driven by enzymatic degradation where degradation rates can be increased by increasing the molecular weight of the polymer[11-12]. It can be hypothesized that upon the introduction of PTMC microspheres[13] into an injectable calcium phosphate cement a controlled degradation pattern will occur without the problems as observed with gelatin. Furthermore, the rubber-like properties of PTMC can possibly also be used to improve the initial mechanical properties of the cement.

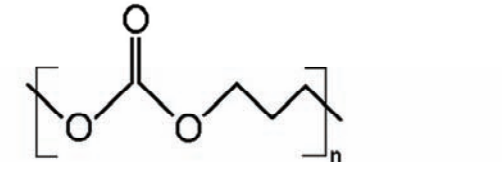



**Figure 1.** Chemical structure of poly(trimethylene carbonate)

In view of the above mentioned, the goal of this study was to formulate a PTMC microsphere/calcium phosphate cement composite (PTMC CPC) with appropriate handling properties and to investigate the mechanical properties and *in vitro* degradation characteristics. Therefore, 12.5 wt % and 25 wt% PTMC CPCs were prepared using PTMC with molecular weights of 52.7 kg·mol$^{-1}$ and 176.2 kg·mol$^{-1}$. Setting time, porosity and mechanical characteristics of the different formulations were determined. For the *in vitro* degradation assay, samples were subjected to enzyme containing medium (lipase) or phosphate buffered saline (PBS). The pH of the medium was monitored and samples were assayed on mass loss, compression strength, E-modulus, chemical consistency (FTIR, XRD) and morphology (SEM).

## 2. Materials and Methods

### *2.1. Materials*

$PTMC_{53}$ ($M_n$ = 52.7 kg·mol$^{-1}$, $M_w$ = 84.1 kg·mol$^{-1}$, PDI = 1.60) and $PTMC_{176}$ ($M_n$ = 176.2 kg·mol$^{-1}$, $M_w$ = 265.2 kg·mol$^{-1}$, PDI = 1.51) were synthesized by ring opening polymerization[13] and used for the preparation of the microspheres. The applied CaP cement is commercially available under the product name Calcibon® and consists of 61 wt% α-TCP, 26 wt% $CaHPO_4$, 10 wt% $CaCO_3$ and 3 wt% pHA. Lipase from *Thermomyces lanuginosus* (EC 3.1.1.3., minimum 100000 units/g, Sigma-Aldrich, St. Louis, USA) was used as PTMC degrading enzyme solution.

### *2.2. Methods*

#### *2.2.1. Preparation of PTMC microspheres*

PTMC microspheres were prepared by a single emulsion method. Briefly, a 1 wt% of $PTMC_{176}$ and respectively 2 wt% of $PTMC_{53}$ solution in dichloromethane was emulsified in an aqueous phase containing 2% PVA. The stirring speeds were respectively 1000 rpm and 800 rpm. After evaporation of dichloromethane, microparticles were solidified, and then purified by centrifugation and re-dispersed in the PVA-containing aqueous phase.

Morphology of the $PTMC_{53}$ and $PTMC_{176}$ microspheres was determined by scanning electron microscopy (SEM, JEOL 6400-LINK AN 10000 at 10 kV); samples were dried on carbon tape and sputtered with gold-palladium prior to measurement. In addition, morphology of the microspheres was also visualized by light microscopy (Leica Microsystems AG, Wetzlar, GER) after which particle size distributions were determined using digital image software (Leica Qwin, Leica).

#### *2.2.2. Preparation of PTMC CPC*

PTMC microsphere/calcium phosphate cements (PTMC CPCs, 12.5 and 25 wt%) of both $PTMC_{53}$ and $PTMC_{176}$ polymers were prepared. For this, the amount of PTMC microspheres inside the suspension was pooled and microsphere suspensions containing 125/250 mg of microspheres were centrifuged

and decanted. Then, 290/260 µl of 1% $Na_2HPO_4$ was added to the microspheres and the resulting suspension was added to 875/750 mg of CPC inside a 2 ml syringe and stirred vigorously for 15s using a Silamat® mixing device (Vivadent, Schaan, Liechtenstein). Cement paste was injected into a Teflon mold (cylinders 4.5*9.0 mm) and samples were left to set at room temperature over night. The applied concentration and amount of liquid hardener for both 12.5 and 25 wt% PTMC CPCs was sufficient to accomplish a fully injectable calcium phosphate cement[4]. After drying, cross-sections of the composites were made and the morphology was determined by SEM.

*2.2.3. Setting time*

Initial and final setting time was assessed using custom available Gillmore needles (ASTM C266). Therefore, a bronze block containing 6 holes (6 mm in diameter, 12 mm in height) was used as a mould and placed in a water bath at body temperature (37˚C). Samples were mixed and injected into the mould in a retrograde fashion, after which the initial and final setting time was determined[4]. Tests were done in triplicate.

*2.2.4. Porosity*

The macro- and total porosity of preset samples was determined. Macroporosity is the porosity generated by the PTMC microspheres and corresponds to the vol% of microspheres. The total porosity is the macroporosity plus the original microporosity of the cement.

To measure these parameters, both PTMC CPC samples and CPC samples of a known volume were placed in an oven at 650°C for 2 h. After burning out the PTMC or moisture, samples were weighed and equation 1 and 2 were used for the derivation of the total porosity and the macroporosity.

$$\varepsilon_{tot} = (1 - \frac{m}{V * \rho_{HAP}}) * 100\% \qquad \textit{Equation 1}$$

$$\varepsilon_{macro} = (1 - \frac{m_{macro}}{m_{micro}}) * 100\% \qquad \textit{Equation 2}$$

*Legend:* $\varepsilon_{tot}$ = total porosity (%), $\varepsilon_{macro}$ = macroporosity (%), m = average mass sample (g, n=3), $m_{macro}$ = average mass macroporous sample (after burning out PTMC) (g, n=3), $m_{micro}$ = average mass microporous sample (CPC) (g, n=3), V = volume sample ($cm^3$), $\rho_{HAP}$ = density hydroxy apatite ($g/cm^3$)

*2.2.5. Mechanical characteristics*

Preset PTMC CPC samples (cylinders, 4.5 mm diameter * 9.0 mm height) were soaked in PBS for 3 days. Subsequently, samples were dried over night in a freeze-dryer, placed in a testing bench (858 MiniBionixII®, MTS Corp., Eden Prairie, MN, USA) and compression strength, E-modulus and strain-at yield (ε) along the vertical axis (height) of the specimens was measured at 0.5 mm/min crosshead speed.

*2.2.6. Degradation assay*

For the degradation assay, preset 12.5/25 wt% $PTMC_{53}$ CPC and $PTMC_{176}$ CPC samples were put in 3 ml of a 1:2 lipase/demineralized water solution or PBS (pH = 7.4) for a total of 6 and 12 weeks respectively. Sample medium was refreshed every week. At day 3, 7, 14, 28, 42 and 84, five samples of each formulation were subjected to analysis as summarized below. CPC samples were taken as reference.

pH measurement

Corresponding to an earlier performed study using PLGA microsphere/calcium phosphate cement composites[4], the pH of the PBS medium was monitored to observe whether conditioning in PBS leads to the formation of acidic compounds.

Mass loss

Sample weight was measured after removal from the incubation medium and freeze-drying over night. Mass loss was determined using Equation 3.

$$R_L = \frac{m_0 - m_n}{m_0} * 100\% \qquad \textit{Equation 3}$$

*Legend:* $R_L$ = mass loss sample on t = n (%), $m_0$ = mass sample on t = 0 (g), $m_n$ = mass sample on t = n (g)

Mechanical characteristics

Compression strength and E-modulus of dried samples was determined by placing the samples in a testing bench as described earlier.

IR spectroscopy

A fraction of the PTMC CPCs samples was grinded and measured by attenuated reflectance Fourier transform infrared spectroscopy (ATR-FTIR, Perkin-Elmer, Fremont, CA, USA). Concentration of PTMC inside the samples was determined analogous to Featherstone et al[14]. In brief, the extinction of a typical PTMC absorption band (C=O, 1750 $cm^{-1}$) and a calcium phosphate absorption band ($PO_4$, 1050 $cm^{-1}$) of the PTMC CPC was determined using Equation 4. The resulting ratio of both extinctions ($E_{C=O}/E_{PO4}$) is linear with the percentage of PTMC inside the cement. Therefore, composites with different contents of PTMC CPC (5, 10, 15, 20, 25 and 30 wt%) were prepared and used to make a standard curve.

$$E = \log \frac{T_{baseline}}{T_{sample\_peak}} \qquad \textit{Equation 4}$$

*Legend:* E = extinction, $T_{baseline}$ = transmission baseline, $T_{sample\text{-}peak}$ = transmission sample peak

Thermogravimetric analysis

Samples for the FTIR standard curve were calibrated using thermogravimetric analysis (TGA 7, Perkin-Elmer). For this assay, 30 mg of sample was placed in the instrument where it was kept at 50°C for 2 min before scanning it from 50°C to 650°C at a rate of 10°C per min. Finally, the sample was kept at 650°C for 2 min before it was cooled down.

X-ray diffraction spectroscopy

The composition of the cement inside the composites was determined by powder X-ray diffraction (XRD, Philips, PW 3710, Almelo, The Netherlands). Therefore, the same grinded samples were used as for FTIR.

Morphology

Cross-sections of the samples were prepared, morphology of the cement and microspheres at the surface and inner part of the composite was monitored at different magnifications by SEM.

*2.2.7. Statistical analysis*

Data were arranged as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat® (GraphPad Software Inc., San Diego, Ca, USA).

## 3. Results

### *3.1. Preparation of PTMC microspheres*

Investigation by SEM and light microscopy revealed that microspheres of both $PTMC_{53}$ and $PTMC_{176}$ polymers showed a spherical structure with a smooth surface. Average size of the microspheres was higher for the $PTMC_{53}$ polymer (28.6 μm, Table 1) compared to the $PTMC_{176}$ polymer (15.3 μm). $PTMC_{176}$ microspheres showed a narrow particle size distribution with a maximum particle size < 40μm, whereas $PTMC_{53}$ microspheres showed a much broader particle size distribution with a maximum size up to 90 μm.

**Table 1.** Physical/mechanical properties PTMC CPCs and CPC

| **Formulation** | **CPC** | **$PTMC_{53}$ CPC** | | **$PTMC_{176}$ CPC** | |
|---|---|---|---|---|---|
| Average size microspheres (μm) | - | 28.63 ± 16.70 | | 15.30 ± 6.21 | |
| PTMC microspheres (wt%) | **0%** | **12.5%** | **25%** | **12.5%** | **25%** |
| Mass samples (mg) | 262.7 ± 6.1 | 200.0 ± 6.3 | 178.9 ± 6.7 | 198.1 ± 6.7 | 177.5 ± 6.7 |
| Initial setting time (s) | 108.3 ± 5.8 | 105.0 ± 0.0 | 183.3 ± 31.8 | 110.0 ± 8.7 | 170.0 ± 34.6 |
| Final setting time (s) | 235.0 ± 31.2 | 480.0 ± 17.3 | > 1200* | 480.0 ± 42.4 | >1200* |
| Total porosity (%) | 40.8 ± 1.3 | 57.3 ± 0.5 | 69.6 ± 0.6 | 62.5 ± 1.5 | 70.0 ± 0.4 |
| Macroporosity (%) | 0 | 27.9 ± 1.4 | 48.7 ± 1.4 | 36.7 ± 1.9 | 49.3 ± 1.3 |
| Compression strength (MPa, n=7) | 64.4 ± 16.6 | 19.0 ± 3.1 | 15.5 ± 2.6 | 23.9 ± 4.8 | 15.3 ± 1.6 |
| E-modulus (GPa, n=7) | 5.44 ± 1.33 | 1.14 ± 0.23 | 0.68 ± 0.13 | 1.60 ± 0.62 | 1.13 ± 0.27 |
| Strain-at-yield (ε, mm, n=7) | 0.186 ± 0.011 | 0.299 ± 0.034 | 0.386 ± 0.116 | 0.215 ± 0.021 | 0.271 ± 0.071 |

* Experiment stopped after 20 min, the specimens harden within 1 day

### *3.2. Preparation/Mechanical properties of PTMC CPC*

Figure 2 shows SEM micrographs of the as-prepared 25 wt% $PTMC_{53}$ CPC and after burning out of the microspheres for the porosity measurements. From the left picture, it can be observed that spheres inside the cement were present with a thin layer of calcium phosphate precipitate onto the surface. The right picture shows that the pores formed by the microspheres are spherical and have a homogenous distribution inside the cement.

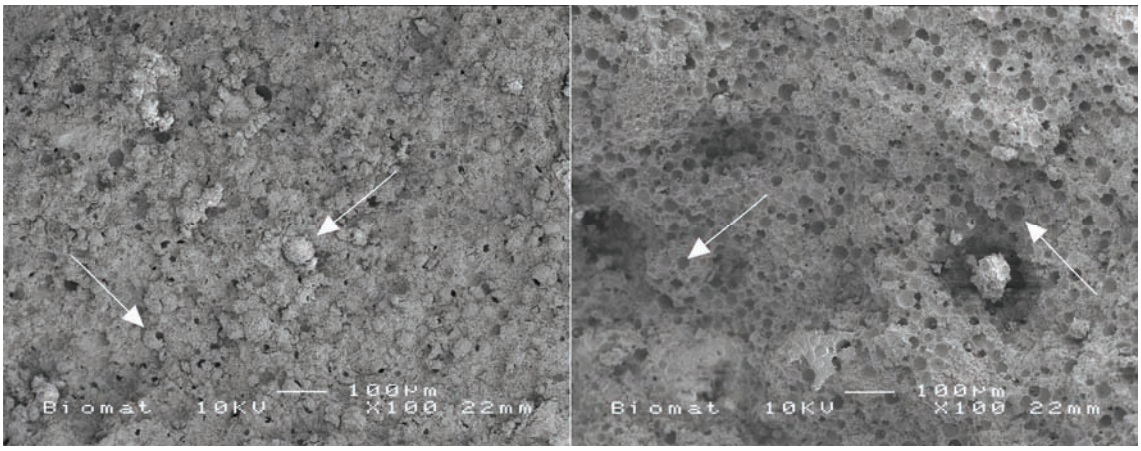



**Figure 2.** Morphology 25 wt% $PTMC_{53}$ CPC as-prepared (left) and after burning out polymer (right), arrows indicate microspheres or pores (original magnification 100×)

Results of the setting time, porosity measurements and mechanical characteristics of the composites are shown in Table 1. Initial setting time of both PTMC CPC composites was similar to the original CPC, whereas final setting time was significantly increased with the 12.5 wt% PTMC CPC and 25 wt% PTMC CPC. Porosity measurements showed a total porosity of ± 60% and 70% with the 12.5 wt% and 25 wt% PTMC CPC, while the microporosity of the original cement reached values up to 40%. Calculated macroporosity (% spheres inside the cement) increased with increasing amount of microspheres, and reached 49% with both 25 wt% PTMC CPCs. For the 12.5 wt% PTMC CPC samples, macroporosity differed significantly ($p<0.05$) between both $PTMC_{53}$ and $PTMC_{176}$ composites.

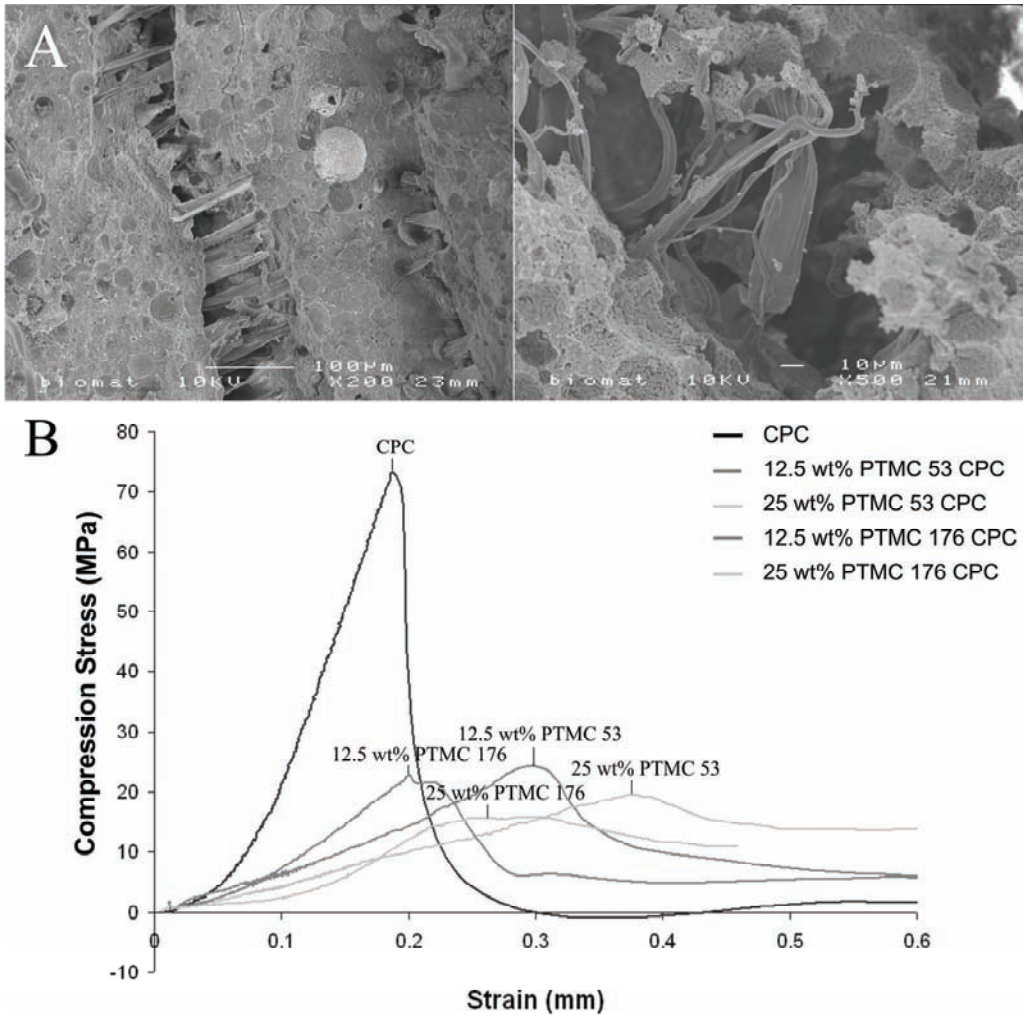



**Figure 3.** A. Stretching of PTMC microspheres at fracture interface (original magnification 200× (left) and 500× (right)), B. Stress-strain curves of CPC and 12.5/25 wt% PTMC CPCs

Compression strength and E-modulus of the PTMC CPCs were significantly lower compared with CPC and decreased with increasing amount of microspheres ($p<0.05$). The E-modulus was dependent on the molecular weight of the PTMC used for the microspheres as significant higher values were observed for $PTMC_{176}$ CPC as compared to $PTMC_{53}$ CPC. Strain-at-yield(ε) of the PTMC CPC samples increased with increasing amount of PTMC microspheres and was significantly higher ($p<0.05$) than the CPC. Also, for $PTMC_{53}$ CPCs a higher ε was observed ($p<0.05$) than the $PTMC_{176}$ CPCs. Furthermore, under high compression PTMC CPC did not fracture into separate smaller pieces but was kept together by the elongated microspheres as depicted in Figure 3A. From stress/strain curves of compression strength measurements (Figure 3B), the same phenomenon was observed with especially the 25 wt% PTMC CPC as compression strength remained at a constant level after reaching yield strengths.

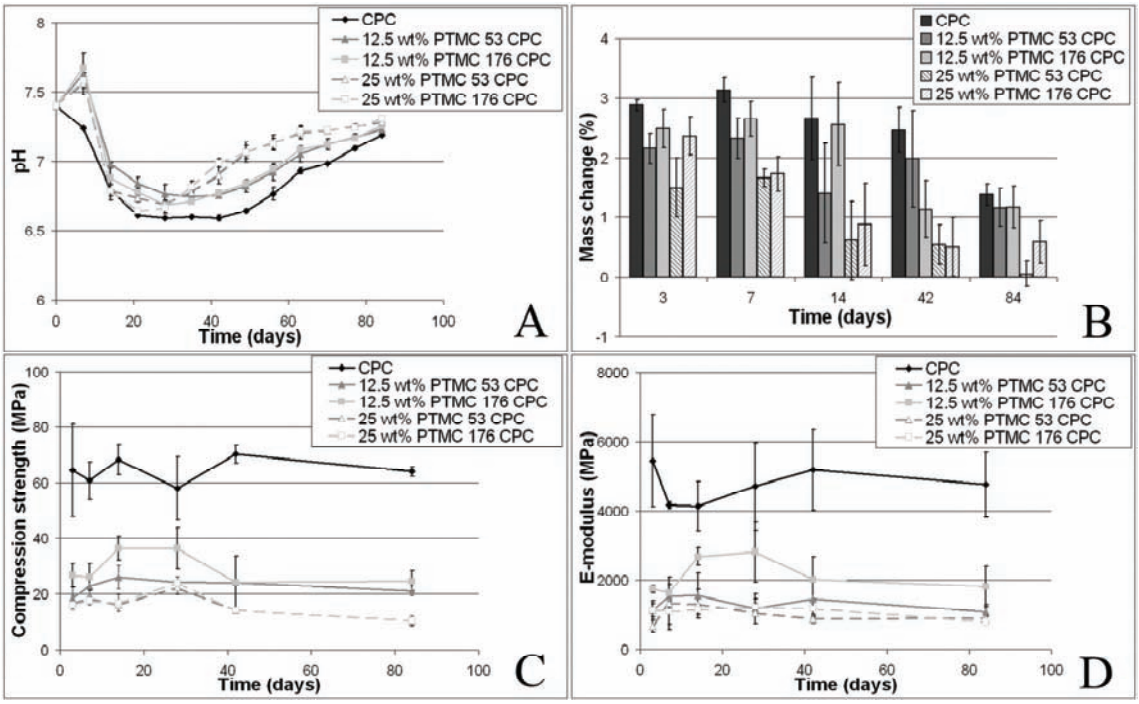



**Figure 4.** Results degradation in PBS; A. pH change of medium per week, B. Mass change in time, C. Compression strength in time, D. E-modulus in time

### *3.3. Degradation in PBS*

Results for the degradation of PTMC CPC in PBS are given in Figures 4-7. Release of acidic products from the CPC into the medium (Figure 4A) resulted in a pH of 6.6 after 21-42 days. Thereafter, the pH decrease was less and the pH slowly returned to its original value after 84 days. The PTMC CPC formulations showed a pattern similar to CPC, but exhibited an overall smaller decrease in pH. For the 25 wt% PTMC CPCs, also a faster increase in pH after 14-28 days was observed. The mass change of the samples in time (Figure 4B) showed an initial increase in mass of 1.5-3.0% for all samples after 3 days incubation time, after which the mass slowly decreased in time. Though changes in mass were small, significant differences ($p<0.05$) between the formulations were observed and the initial mass increase was in the following order: CPC > 12.5 wt% PTMC CPC > 25 wt% PTMC CPC. Compression strength and E-modulus of the PTMC CPC and CPC samples (Figure 4C-D) was constant over time, but, at t = 14 and 28 days values with the 12.5 wt% $PTMC_{53}$ CPC were slightly higher. Furthermore, compression strength of the 25 wt% PTMC CPCs showed a significant decrease after 84 days ($p<0.005$).

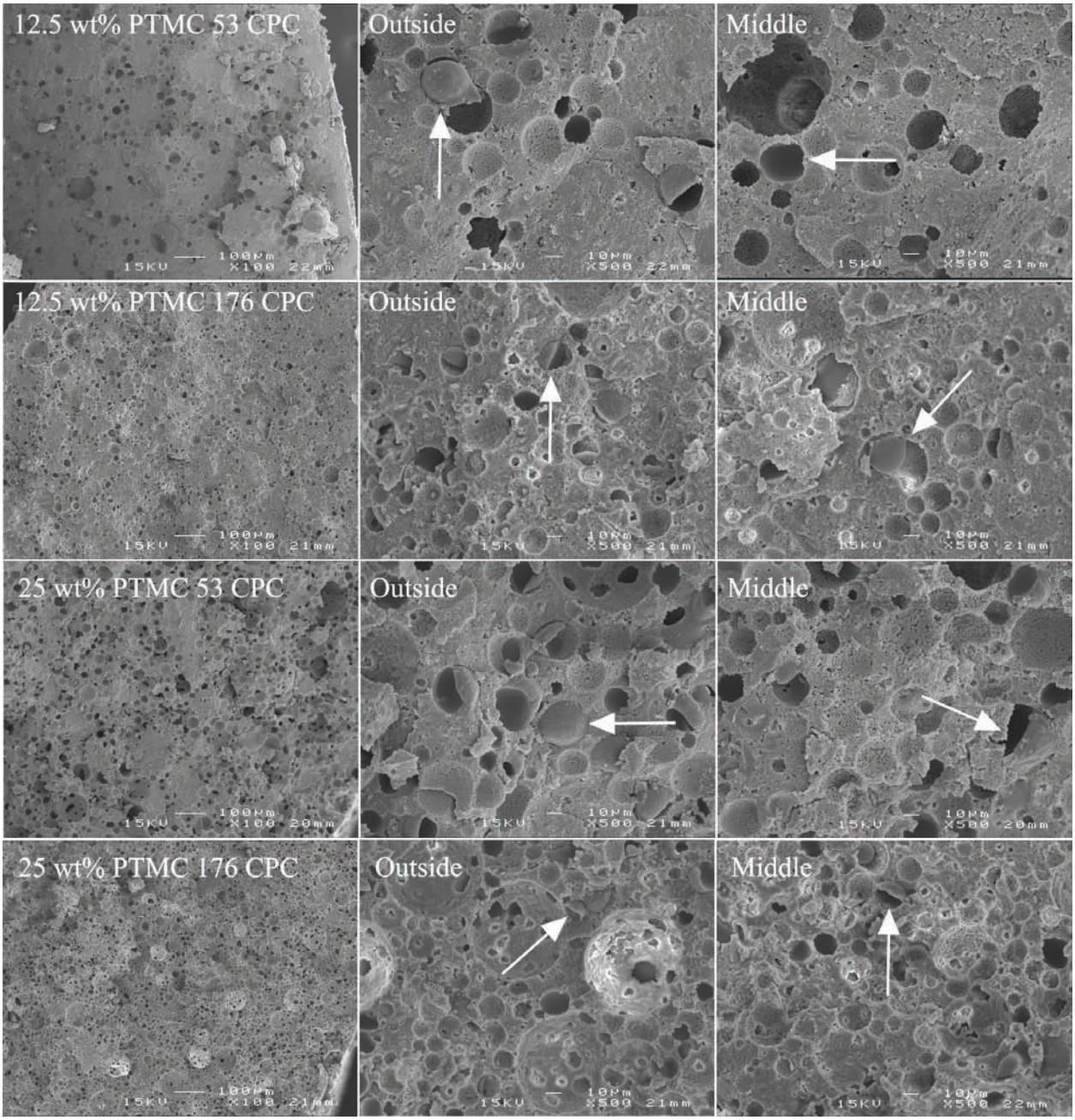



**Figure 5.** SEM-micrographs of 12.5 wt% and 25 wt% $PTMC_{53}$ CPC / $PTMC_{176}$ CPC samples at t = 42 days in PBS, arrows denote PTMC microspheres/shells (orig. magn. 100× and 500×)

SEM-micrographs of the PTMC CPC composites (Figure 5) show spherical pores and microsphere structures that are scattered evenly through the cement. For the 12.5 wt% PTMC CPC, these pores/spheres are isolated inside the cement, whereas for 25 wt% PTMC CPC there is a tight packing. However, interconnections between pores are very scarce. Due to the different microsphere sizes, there is a slight difference in pore size between the $PTMC_{53}$ CPC and $PTMC_{176}$ CPC. No clear differences in morphology were observed between samples taken at t = 42 days and other time points (data not shown).

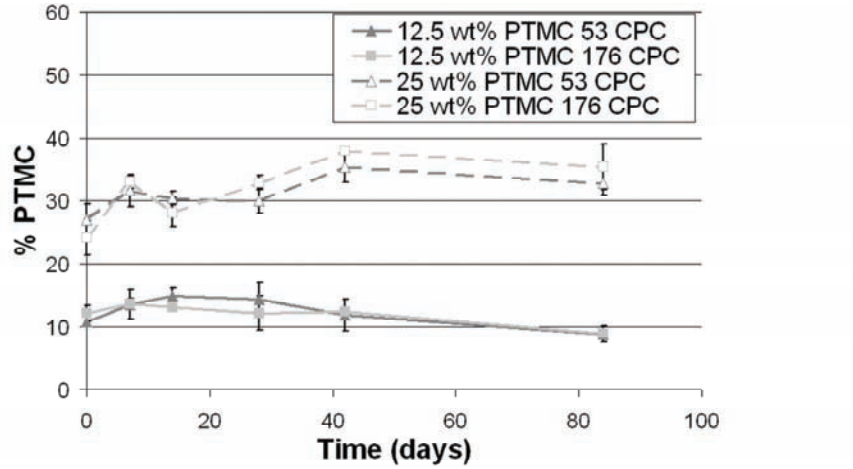



**Figure 6.** Weight% of PTMC in samples as measured by FTIR as function of incubation time in PBS

FTIR was performed to quantitatively determine the amount of PTMC inside the composites. Standard curves that were analyzed with FTIR (and calibrated with TGA) showed a linear relationship between the $E_{C=O}/E_{PO4}$ ratio and PTMC content ($r^2 > 0.98$) for both $PTMC_{53}$ and $PTMC_{176}$ polymers. Using these standard curves, the percentage of PTMC inside the composites was calculated as is shown in Figure 6. In PBS, both $PTMC_{53}$ and $PTMC_{176}$ CPCs showed a similar trend and a slight increase in %PTMC over time with the 25 wt% PTMC CPCs was determined, whereas the 12.5 wt% PTMC CPCs showed a slight decrease of PTMC content after 84 days. Investigation of the cement structure of the PTMC CPCs by X-ray diffraction (Figure 7, PBS) revealed that the cement starting compounds (α-TCP, monetite) disappeared within 14 days of incubation, with only the endproduct (apatite) remaining.

### *3.4. Degradation in lipase solution*

Results of the degradation of PTMC CPC in lipase solution are depicted in Figures 7-11. Mass change of the PTMC CPCs in time (Figure 8A) showed different curves for both 12.5 wt% and 25 wt% PTMC CPCs. For 12.5 wt% PTMC CPC, there was an initial increase in mass of 2% after 3 days that significantly increased to 3.5% after 42 days ($p<0.005$). The 25 wt% PTMC CPC samples showed a decrease in mass with 1.1-1.7 wt% after 7 days after which also a slight increase in mass was observed. Compression strength (Figure 8B) of the 25 wt% PTMC CPC (5-10 MPa) at most time points was significantly lower than for the 12.5 wt% PTMC CPC (15-22 MPa) and did not show a clear trend in time. The E-modulus of both systems (data not shown) showed a similar trend as the compression strength, though less significant differences were observed.

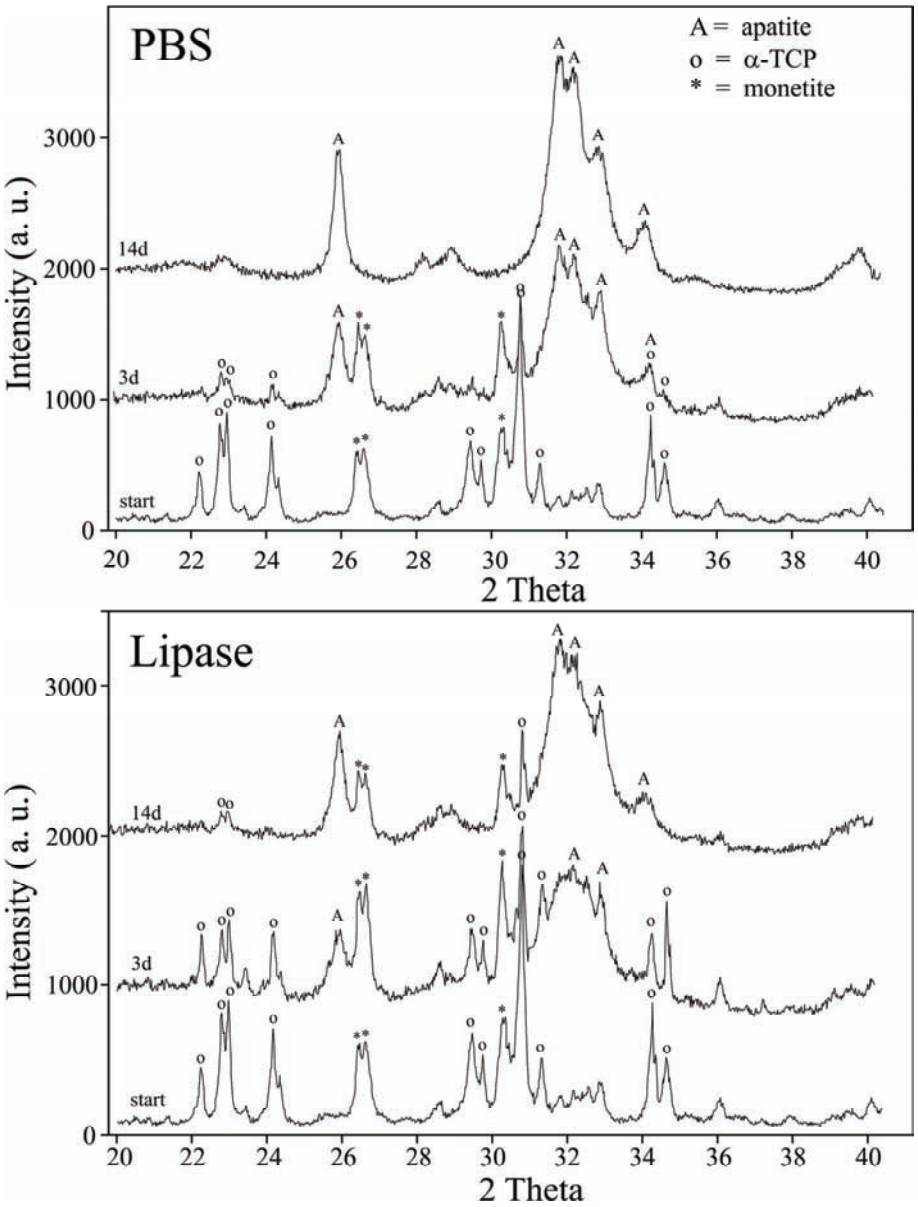



**Figure 7.** XRD spectra of cement starting material and 25 wt% $PTMC_{53}$ CPC after 3 and 14 days in PBS and lipase solution, legend figures: A = apatite, o= α-TCP, * = monetite

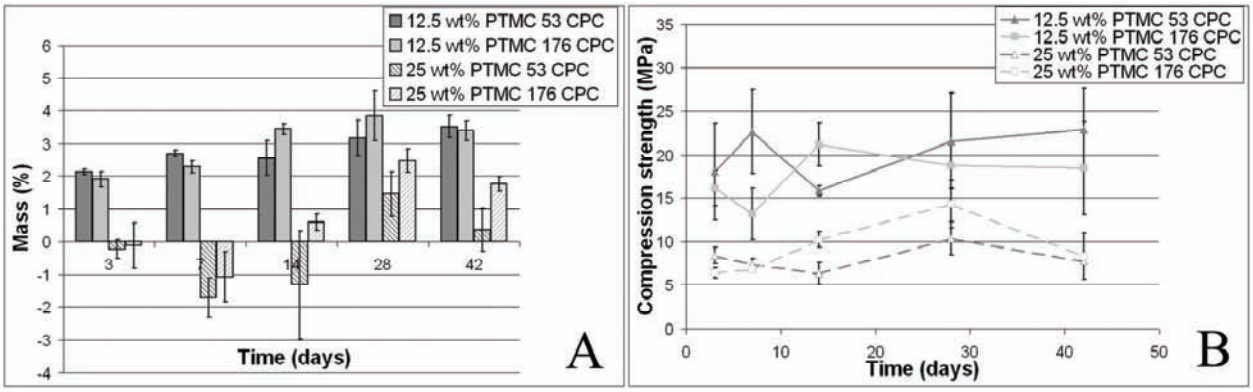



**Figure 8.** Degradation of PTMC CPC in lipase solution; A. Mass change in time, B. Compression strength in time

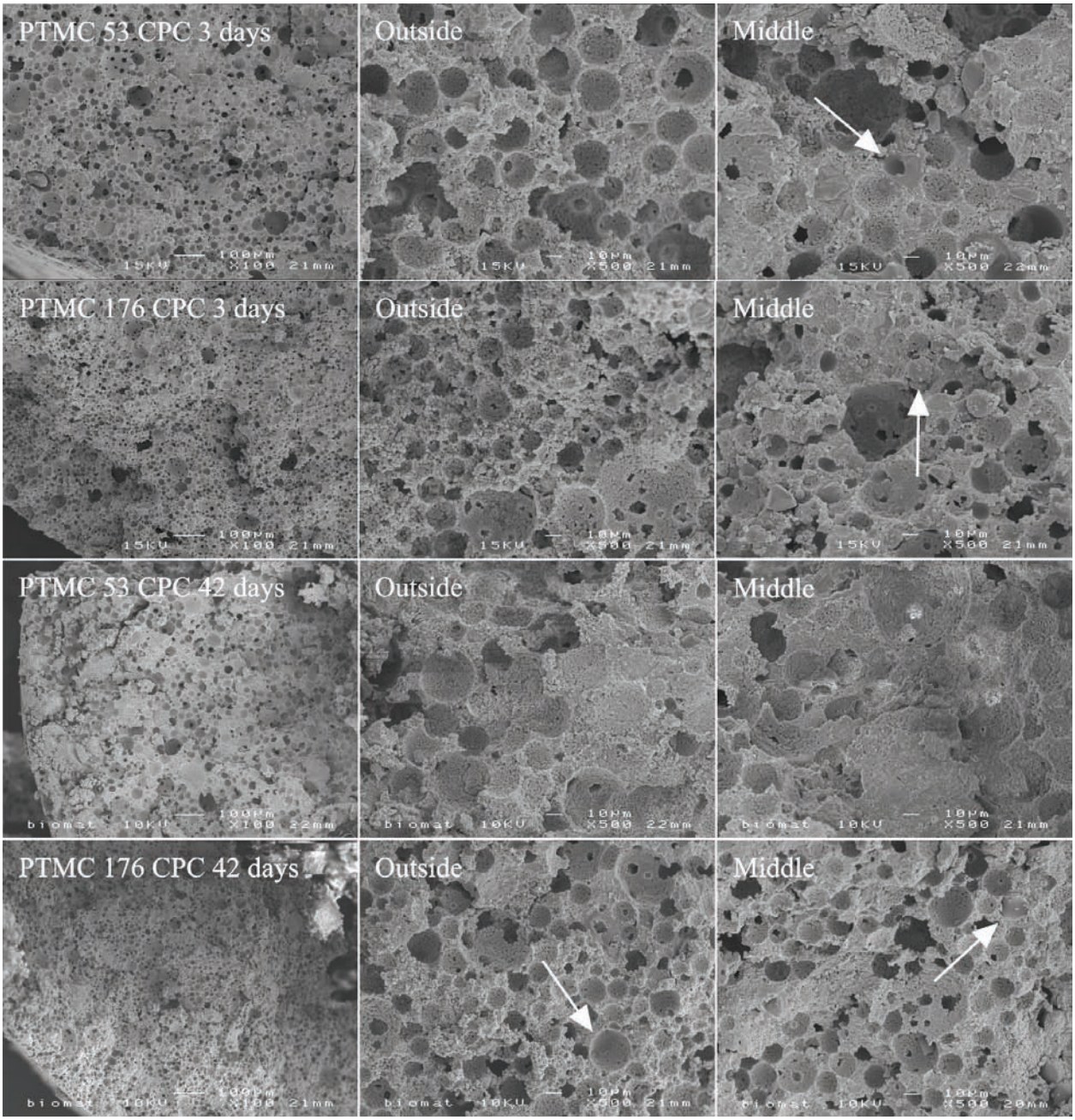



**Figure 9.** SEM-micrographs of 25 wt% $PTMC_{53}$ CPC and $PTMC_{176}$ CPC samples at t = 3, 42 days in lipase solution, arrows denote PTMC microspheres/shells (orig. magn. 100× and 500×)

SEM micrographs of 25 wt% PTMC CPCs (Figure 9) at day 3 show an open porous structure at the outside of the samples, whereas in the inside microspheres were present. A similar morphology was observed after 42 days, though the amount of microspheres present in the middle was markedly reduced. Conversely, with the 12.5 wt% PTMC CPC (Figure 10) a lot of microspheres were visible at t = 3 and 42 days and only at the upper 200μm of the samples the amount of microspheres was reduced. Furthermore, the cement microstructure of especially the 25 wt% PTMC CPCs showed a strikingly high porosity.

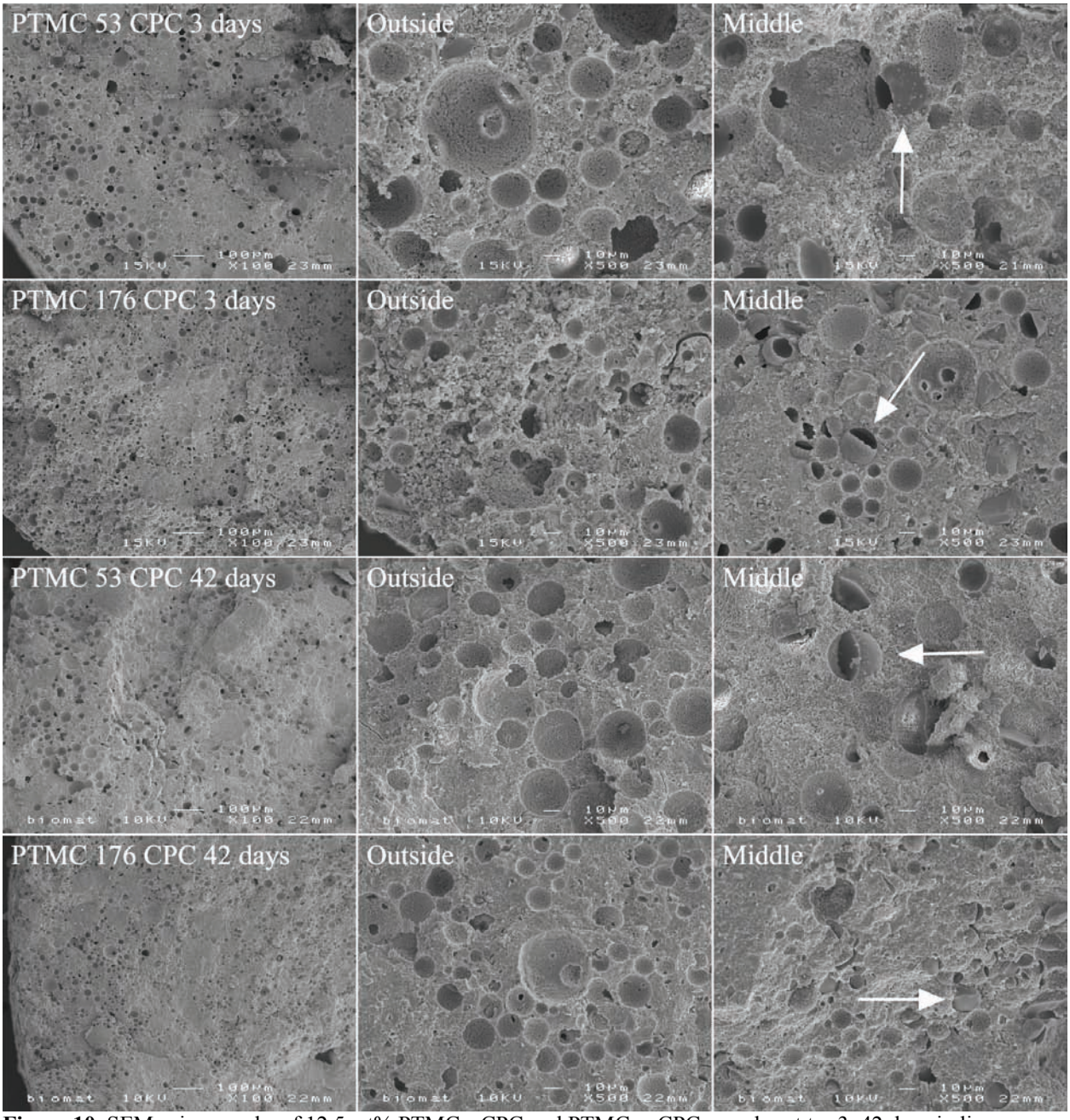



**Figure 10.** SEM-micrographs of 12.5 wt% $PTMC_{53}$ CPC and $PTMC_{176}$ CPC samples at t = 3, 42 days in lipase solution, arrows denote PTMC microspheres/shells (orig. magn. 100× and 500×)

Results from FTIR spectroscopy are given in Figure 11A-B. Figure 11A gives a representation of the acquired IR spectra with 25wt% $PTMC_{53}$ CPC after t = 7, 14, 28 and 42 days. A distinct decrease of peak intensity of all separate PTMC peaks was observed with increasing degradation times, whereas the $PO_4$-peak of the CaP cement remained constant. Furthermore, a peak at 1650 $cm^{-1}$ was present, that was absent for PTMC CPC in PBS medium, showing a slight increase in time. As this wavelength corresponds to the C=O stretch of proteins/amides, this peak may indicate the presence of lipase inside the scaffolds. Figure 11B shows the wt% of PTMC inside the composite as a function of degradation time. For both 25 wt% PTMC CPC samples, a significant decrease in the wt% of PTMC was observed from 25 wt% to 5-8 wt% at 42 days that was faster with the $PTMC_{53}$ CPC. The composition of the

12.5 wt% PTMC CPCs did vary less in time, although also a small decrease in the wt% of PTMC was noted. Finally, the analysis of the cement by XRD (Figure 7, lipase) revealed that after 14 days of incubation peaks of monetite and α-TCP were still present, though also here cement was converted into apatite.

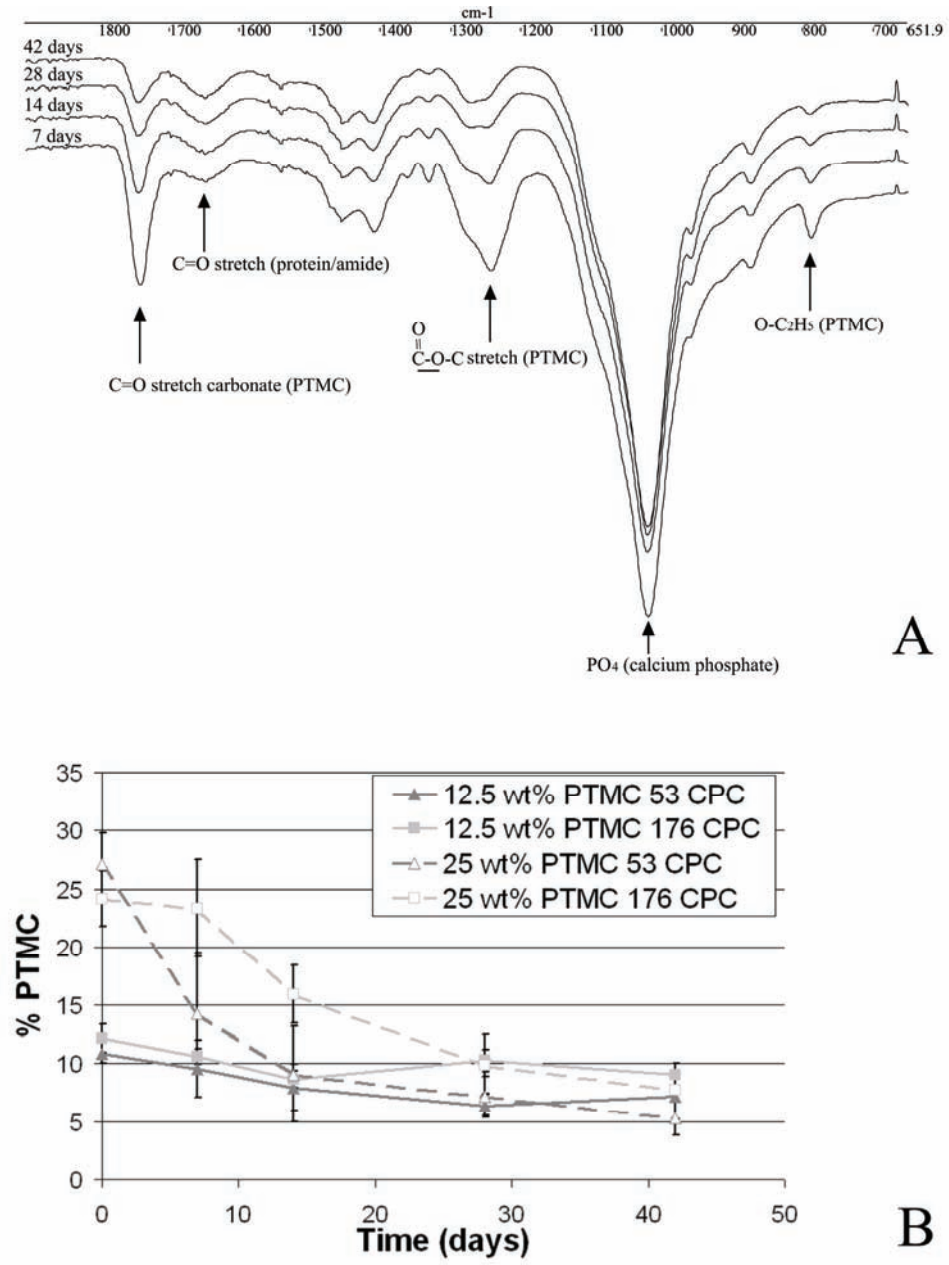



**Figure 11.** A. IR-spectra of 25wt% $PTMC_{53}$ CPC in lipase solution after t = 7, 14, 28 and 42 days, B. Weight% of PTMC in samples as measured by FTIR as function of incubation time in lipase solution

## 4. Discussion

In this study, physical/mechanical properties and *in vitro* degradation characteristics of injectable calcium phosphate cement with incorporated PTMC microspheres was investigated. Therefore, 12.5 wt% and 25 wt% $PTMC_{53}$ and $PTMC_{176}$ microsphere/ calcium phosphate cement composites were formulated, which were tested on setting properties, mechanical strength and macroporosity.

Furthermore, preset samples were incubated in PBS/lipase and assayed for weight loss, mechanical strength, morphology and composition (XRD, FTIR).

The used poly(trimethylene carbonate) is a polymer with a glass transition temperature ($T_g$) ~ -20°C[15]. Consequently, at room and body temperature PTMC behaves as a rubber-like polymer. During preparation of polymeric microsphere CPC composites, (freeze-)dried microspheres are usually mixed with calcium phosphate cement powder to obtain a good distribution of the microspheres, after which liquid hardener is added[4]. Due to its low $T_g$, freeze-dried PTMC microspheres agglomerate and therefore cannot be mixed with the cement powder. Therefore, to obtain a PTMC CPC, microspheres were added to the liquid hardener during cement preparation as described in the materials and methods section, which resulted in a well distributed microsphere composite. In agreement with previous studies using PLGA and gelatin microspheres[4-5], setting time was increased upon the addition of PTMC microspheres. When compared to conventional CPC[16] setting time increased due to an increase in liquid/powder ratio as extra aqueous phase was still present after carefully decanting the PVA solution above the PTMC microspheres. Also with PLGA microspheres[4] and gelatin microspheres[5] extra aqueous phase was present to improve the injectability of the composite (PLGA) or as a result of the preparation method (pre-swollen gelatin microspheres). Another parameter that influenced the setting of the PTMC CPC is the large volume of microspheres of especially 25 wt% PTMC CPC that hampers the entanglement of calcium phosphate crystals (setting process)[17]. Furthermore, even after the cement has set, imprints of the Gilmore needle were still visible as a result of plastic deformation of the composite[18].

For the degradation study PBS as well as a lipase solution were used. Though in PBS no degradation of the microspheres is expected, this study was necessary to compare the current results with microsphere CPC specimens in previous experiments[4-6]. Comparable to *in vitro* studies with pure PTMC specimens[11], the lipase solution was used to determine whether microspheres within a calcium phosphate scaffold can degrade in the presence of a PTMC degrading enzyme. Although it has to be emphasized that in a bony environment the concentration of lipase will be much lower, implantation of solid PTMC scaffolds in the tibia and femur of rabbits[11] also resulted in a sustained surface erosion of the polymer indicating the presence of PTMC degrading enzymes.

Results of the pH assay for the degradation in PBS showed that a higher amount of incorporated PTMC microspheres results in a smaller pH decrease, indicating that the cement is the major source of acidic (degradation) products[4, 19]. Furthermore, results of the PBS study showed that there was no sign of PTMC degradation as an initial mass increase of 1.5-2.5% was measured, while the compression strength/E-modulus remained constant during the first 6 weeks of incubation. Nevertheless, after the first week the mass of the samples gradually decreased in time and also the compression strength decreased slightly after 12 weeks of incubation. As the same trend is observed with the CPC control, cement dissolution/degradation over time is the most plausible explanation. The

initial mass increase was also observed in previous studies[5, 6] and is caused by precipitation of salts from the PBS solution.

SEM pictures of the 25 wt% PTMC CPC specimens that were incubated in lipase solution showed an interconnected structure of macropores, which confirms that the microspheres have been degraded. At early time points (t = 3 days) this porosity was mostly present at the outer parts whereas at later time points also at the inner radius the amount of microspheres was reduced. This result is comparable to that for the gelatin microsphere CPC in a previous experiment[6] and indicates a similar pattern with microspheres degrading from the outer to the inner part of the implants. Also results with IR showed a slow decrease in the wt% of PTMC within the composite, confirming a sustained degradation of the PTMC polymer. Both analyses showed that degradation of PTMC (microspheres) in the 12.5 wt% composites was much slower. This corresponds to studies with gelatin microspheres[5-6], and is caused by the low or non-existent interconnectivity of the microspheres that delays the penetration of lipase into the composite.

A striking observation in the IR spectra is that for 25 wt% PTMC CPC, the $PTMC_{53}$ microspheres inside the cement degrade faster than the $PTMC_{176}$ microspheres, whereas the highest molecular weight PTMC degrades faster in separate degradation experiments[11]. As $PTMC_{176}$ microspheres were significantly smaller than the $PTMC_{53}$ microspheres, they also contained a relatively higher amount of hydrophilic PVA at the surface[20]. This may form a barrier for enzymatic degradation as the enzymes are more active at hydrophobic surfaces. The difference in size of the $PTMC_{53}$ and $PTMC_{176}$ microspheres was related to different parameters that were applied in their preparation.

The results from the mass assay do not corroborate with the IR and SEM results. If mass decrease would be in agreement with these results, the mass of 25 wt% PTMC CPC should have decreased with 15-20% after 6 weeks. However, only an initial decrease in mass was observed followed by a slow increase in mass. The reason for this phenomenon is that within this system there are two processes occurring at the same time, i.e. degradation of PTMC microspheres and adsorption of organic components (lipase) from the medium. Next to the appearance of a typical protein/amide-peak in IR spectra[21], occasionally a layer of precipitate at the surface of the sample was observed in SEM and the cement itself turned from white to yellow (color of lipase medium) in time.

For both 12.5 wt% and 25 wt% PTMC CPC either in PBS or in lipase solution, the compression strength/E-modulus remained constant in time. While in PBS there is no PTMC degradation, the compression strength of the PTMC CPC is expected to be constant. However, in lipase solution 25 wt% PTMC CPC should show a decrease in compression strength[4-6] as the polymer is slowly degrading. Comparing the compression strengths of samples exposed to lipase solution with that of samples in PBS, 25 wt% PTMC CPC samples (but also 12.5 wt% PTMC CPC) have a lower value from the beginning (3 days) in lipase and no gradual decrease. This is probably due to the fact that the PTMC microspheres degrade by surface erosion[11] thereby detaching themselves from the surrounding cement. As a consequence, the compression strength decreases immediately after the start of

microsphere degradation. A slower cement conversion rate in lipase solution as can be suggested from Figure 7 is also an explanation for a lower initial mechanical strength, though in both media the cement was highly converted into apatite after 14 days.

Finally, the introduction of a rubber-like PTMC into a calcium phosphate cement for tissue engineering possibly can be used to improve the mechanical properties of the cement, i.e. overcome brittleness[22], as a higher strain-at-yield was obtained with an increasing percentage of PTMC. Also the toughness of the cement increased as composites did not fracture after reaching the yield strength but were kept together by the elongated microspheres. Similar mechanical characteristics are also observed using fiber-containing calcium phosphate cements[23-24]. Compared to these fiber-containing cements, microsphere/calcium phosphate cement composites are more easily to process with respect to injectability and polymer distribution and therefore can form an attractive alternative.

**5. Conclusion**

Calcium phosphate cements incorporated with well distributed PTMC microspheres were formulated and showed initial setting times of ± 2-3 min and compression strengths of 15-24 MPa. Gradual degradation of the incorporated microspheres occurred from the outer to the inner part of the composite when they were incubated in lipase solution, but not in PBS. The incorporation of PTMC microspheres in CPC improved the toughness of the material, preventing it from fracturing into smaller pieces.

*Acknowledgements*

Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) R.Z. LeGeros, Properties of osteoconductive biomaterials: calcium phosphates, Clin Orthop Rel Res. 395 (2002) 81-98

2) E.M. Ooms, J.G.C. Wolke, M.T. van de Heuvel, B. Jeschke, J.A. Jansen, Histological evaluation of the bone response to calcium phosphate cement implanted in cortical bone, Biomaterials. 24(6) (2003) 989-1000

3) P.Q. Ruhé, H.C. Kroese-Deutman, J.G.C. Wolke, P.H.M. Spauwen, J.A. Jansen, Bone inductive properties of rhBMP-2 loaded porous calcium phosphate cement implants in cranial defects in rabbits, Biomaterials. 25 (2004) 2123-2132

4) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cemens: physical properties and degradation characteristics, J Biomat Sci Polym Ed. 17(9) (2006) 1057-1074

5) W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen, Introduction of gelatin microspheres into an injectable calcium phosphate cement, J Biomed Mater Res Part A. Published online January 11, 2008

6) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Porcine gelatin microsphere/ calcium phosphate cement composites: *in vitro* degradation and drug release, *Submitted to J Biomed Mater Res Part B: Appl Biomat*

7) P.Q. Ruhé, O.C. Boerman, F.G. Russel, P.H. Spauwen, A.G. Mikos, J.A. Jansen, Controlled release of rhBMP-2 loaded poly(dl-lactic-co-glycolic acid)/calcium phosphate cement composites in vivo, J Control Release. 106(1-2) (2005) 162-171

8) T.A. Holland, Y. Tabata, A.G. Mikos, Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering, J Control Release. 101(1-3) (2005) 111-125

9) A. Bigi, E. Boanini, S. Panzavolta, N. Roveri, K. Rubini, Bonelike apatite growth on hydroxyapatite-gelatin sponges from simulated body fluid, J Biomed Mater Res Part A. 59 (2002) 709-714

10) A-C. Albertsson, M. Eklund, Infuence of molecular structure on the degradation mechanism of degradable polymers: in vitro degradation of poly(trimethylene carbonate), poly(trimethylene carbonate-co-caprolactone) and poly(adipic anhydride), J Appl Polym Sci. 57 (1995) 87-103

11) Z. Zhang, R. Kuijer, S.K. Bulstra, D.W. Grijpma, J. Feijen, The in vivo and in vitro behavior of poly(trimethylene carbonate), Biomaterials. 27(9) (2006) 1741-1748

12) Z. Zhang, S. Zou, G.J. Vansco, D.W. Grijpma, J. Feijen, Enzymatic surface erosion of poly(trimethylene carbonate) films studied by atomic force microscopy, Biomacromolecules. 6(6) (2005) 3404-3409

13) Z. Zhang, M.A. Foks, D.W. Grijpma, J. Feijen, PTMC and MPEG-PTMC microparticles for hydrophilic drug delivery, J Control Release. 101(1-3) (2005) 392-394

14) J.D.B. Featherstone, S. Pearson, R.Z. LeGeros, An Infrared method for quantification of carbonate in carbonated apatites, Caries Res. 18 (1984) 63-66

15) A.P. Pêgo, A.A. Poot, D.W. Grijpma, J. Feijen, Physical properties of high molecular weight 1,3-trimethylene carbonate and D,L-lactide copolymers, J Mater Sci Mater Med. 14(9) (2003) 767-773

16) I. Khairoun, M.G. Boltong, F.C.M. Driessens, J.A. Planell, Effect of calcium carbonate on clinical compliance of apatitic calcium phosphate bone cement, J Biomed Mater Res Part A. 38 (1997) 356-360

17) C. Liu, W. Shen, G. Yanfang, L. Hu, Mechanism of the hardening process for a hydroxyapatite cement, J Biomed Mater Res Part A. 35 (1997) 75-80

18) Z. Zhang, D.W. Grijpma, J. Feijen, Triblock copolymers based on 1,3-trimethylene carbonate and lactide as biodegradable thermoplastic elastomers, Macromol Chem Phys. 205 (2004) 867-875

19) D.P. Link, J. van den Dolder, J.G.C. Wolke, J.A. Jansen, The cytocompatibility and early osteogenic characteristics of an injectable calcium phosphate cement, Tissue Eng 13(3) (2007) 493-500

20) J. Panyam, M.M. Dali, S.K. Sahoo, W. Ma, S.S. Chakravarthi, G.L. Amidon, R.J. Levy, V. Labhasetwar, Polymer degradation and in vitro release of a model protein from poly(D,L-lactide-*co*-glycolide) nano- and microparticles, J Control Release. 92 (2003) 173-187

21) C.J. Pouchert (Editor). The Aldrich library of infrared spectra Edition III. 1981 page 435

22) W.J.E.M. Habraken, J.G.C. Wolke, J.A. Jansen, Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering, Adv Drug Deliv Rev. 59(4-5) (2007) 234-248

23) F. Buchanan, L. Gallagher, V. Jack, N. Dunne, Short-fibre reinforcement of calcium phosphate bone cement, Proc Inst Mech Eng. 221(2) (2007) 203-211

24) H.H. Xu, J.B. Quinn, Calcium phosphate cement containing resorbable fibers for short-term reinforcement and macroporosity, Biomaterials. 23(1) (2002) 193-202

# CHAPTER 8

## IN VIVO DEGRADABILITY OF CALCIUM PHOSPHATE CEMENT WITH INCORPORATED BIODEGRADABLE MICROSPHERES

*W.J.E.M. Habraken, H.B. Liao, Z. Zheng, J.G.C. Wolke, D.W. Grijpma, A.G. Mikos, J. Feijen, J.A. Jansen*

Submitted to: Adv Mater. (2008)

## 1. Introduction

Most of the currently available calcium phosphate cements (CPCs) convert after application in a bone defect into apatite, which is a calcium phosphate phase that is similar to the inorganic part of bone. A disadvantage of this end-product is its high stability and therefore this material shows only a slow osteoclastic depending resorption in time[1]. Although, a faster degrading brushite cement[2-3] can be selected to solve this problem, another approach is the introduction of macroporosity[4] into the apatite cement. The consequential increase of surface area results in an increased resorption rate of the cement. Macroporosity can be generated by the addition of foaming agents to the cement such as sodium bicarbonate[5-6], bovine serum albumin[7], water soluble crystals[8] and by the introduction of biodegradadable microspheres[9-11]. This last method has the advantage that the mechanical strength of the microspheres contributes to the initial strength of the ceramic scaffold[12]. Additionally, these microspheres can be applied as drug delivery vehicle for osteoinductive growth factors[10].

Previous studies have already shown that indeed injectable and setting CPCs can be produced upon the introduction of microspheres composed of poly(lactic-*co*-glycolic acid) (PLGA)[12], gelatin[13] and poly(trimethylene carbonate) (PTMC)[14]. *In vitro* incubation of these composites in phosphate buffered saline or enzyme-containing media[12, 14, 15] results in an interconnected, macroporous calcium phosphate matrix after microsphere degradation. The *in vitro* degradation mechanisms of the different microsphere/calcium phosphate formulations in these studies were varied by using polymers with unique degradation or erosion mechanisms (Table 1)[16]. For example, gelatin and PTMC microsphere/CPC composites[14, 15] were found to show a gradual degradation of the microspheres from the outer to the inner parts of the composite as the cement delays enzyme diffusion through the material. On the other hand, in PLGA microsphere containing cements[12], microspheres degraded throughout the cement as a result of hydrolytic cleavage of the polymer chains. Furthermore, compared to the bulk erosion mechanism of PLGA and gelatin microspheres[12, 15], surface erosion of the PTMC microspheres[16] resulted in a fast decrease of compression strength as they detached from the cement matrix[14]. In an *in vivo* situation, conditions are different regarding fluid flow, buffer capacity and enzyme activity[17], and therefore it can be hypothesized that the microsphere composites degrade then in a different manner.

**Table 1.** Degradation/erosion pattern biodegradable polymers

| | PLGA | Gelatin | PTMC |
|---|---|---|---|
| Degradation | Hydrolysis | Enzymatic | Enzymatic |
| Erosion | Bulk erosion | Bulk erosion | Surface erosion |

The goal of this study is to investigate the *in vivo* degradation behavior and tissue response of microsphere/calcium phosphate cement composites (microsphere CPCs) with different microsphere degradation and erosion mechanisms. As a model, microsphere CPCs composed of calcium phosphate cement containing either PLGA microspheres, gelatin microspheres or PTMC microspheres were

formulated and implanted subcutaneously in the back of New Zealand white rabbits. Additionally, to investigate the influence of the type of gelatin, both porcine (type A) and bovine (type B) gelatin microspheres were introduced into the cement. PTMC microspheres with a relatively low molecular weight of 40.0 $kg \cdot mol^{-1}$ and 444.2 $kg \cdot mol^{-1}$ were incorporated into the cement as previous experiments[16] showed that higher molecular weight PTMC exhibits a higher erosion rate. Finally, also a composite of cement with incorporated gelatin and PLGA microspheres was formulated to investigate whether the degradation of one type of microspheres influenced the degradation of the other type of microspheres.

## 2. Materials and Methods

### *2.1. Materials*

Poly(lactic-co-glycolic acid) (PLGA, L/G : 54/46, $M_n$ = 34.7 $kg \cdot mol^{-1}$, $M_w$ = 54.2 $kg \cdot mol^{-1}$, PDI = 1.56, Purac, Gorinchem, The Netherlands), gelatin (porcine (type A): pI = 7.0-9.0; bovine (type B): pI = 4.5-5.0, both Acros, Geel, Belgium) and poly(trimethylene carbonate)[16] (LMW PTMC: $M_n$ = 40.0 $kg \cdot mol^{-1}$, $M_w$ = 68.7 $kg \cdot mol^{-1}$, PDI = 1.72, HMW PTMC: $M_n$ = 444.2 $kg \cdot mol^{-1}$, $M_w$ = 599.0 $kg \cdot mol^{-1}$, PDI =1.35) were used for the preparation of the microspheres. Poly(vinyl alcohol) (PVA, 88% hydrolyzed, $M_w$ = 22 $kg \cdot mol^{-1}$, Acros) was used as stabilizer during microsphere preparation. The calcium phosphate cement (Calcibon®, Biomet Merck, Darmstadt, Germany) is commercially available and consists of 61% α-TCP, 26% DCPA, 10% $CaCO_3$ and 3% pHA. The applied cement liquid was a 1% w/v $Na_2HPO_4$ solution in demineralized water ($ddH_2O$).

### *2.2. Methods*

#### *2.2.1. Preparation of PLGA microspheres*

PLGA microspheres were prepared by a water-in-oil-in-water (w/o/w)-double emulsion solvent evaporation technique[18]. 1.0 g of PLGA was dissolved in 4 ml of dichloromethane (DCM) in a 50 ml glass test tube. 500µl of demineralized water ($ddH_2O$) was added while vortexing vigorously for 1 min, subsequently adding 6 ml of a 0.3% w/v poly(vinyl alcohol) (PVA) solution in $ddH_2O$ and continuing vortexing for another 1 min. The content of the tube was transferred to a 1000 ml beaker and stirred by a magnetic stirring bar, followed by adding another 394 ml of 0.3% PVA and 400 ml of a 2% isopropyl alcohol (IPA) solution. The suspension was stirred for 1 hr, the formed microspheres were allowed to settle for 15 min and the solution was decanted. The remaining suspension was centrifuged and the microspheres were washed three times with $ddH_2O$. Finally the microspheres were frozen, freeze-dried for 24 hr (-40°C, 0.13 mbar) and stored under argon at -20°C.

*2.2.2. Preparation of gelatin microspheres*

Gelatin microspheres were prepared using an established technique[19]. Briefly, 2.5 g of gelatin type A (GELA) / gelatin type B (GELB) was dissolved in 25 ml dd$H_2O$ at an elevated temperature (30 min at 60°C). The resulting clear solution was added slowly (10 ml pipette) to a 250 ml three-necked round bottom flask containing 125 ml olive oil (Acros) while stirring at 500 rpm (Teflon upper stirrer). The round bottomed flask was cooled using an ice bath and after 30 min, 50 ml of chilled acetone (4°C) and glutaraldehyde (0.5 ml = 6.25 mM) was added gently. The solution was stirred for another 1h, after which the microspheres were collected by filtration (paper filter, D3, Schott Duran, Mainz, Germany) and washed several times with acetone (~1 L) to remove residual olive oil. Thereafter, the microspheres were stored in a vacuum exicator.

*2.2.3. Preparation of PTMC microspheres*

PTMC microspheres were prepared by a single emulsion method[20]. 1% w/v of HMW PTMC and respectively 2% w/v of LMW PTMC solutions in dichloromethane were emulsified in an aqueous phase containing 2% PVA. The stirring speeds were respectively 1000 rpm and 800 rpm. After evaporation of dichloromethane, microparticles were formed, which were purified by centrifugation and redispersed in the PVA-containing aqueous phase.

*2.2.4. Preparation of microsphere/calcium phosphate cement composites*

Before the microsphere/calcium phosphate cement composites were prepared, calcium phosphate cement powder was sterilized by gamma-irradiation (25 kGy, Isotron, Ede, The Netherlands). Microspheres were sterilized by UV (254 nm) to prohibit extensive degradation of the PLGA[21] or crosslinking of the PTMC polymer. Subsequently, cement composites were prepared and stored in a sterile environment. Briefly, for the preparation of the microsphere/calcium phosphate cement composites, (freeze-)dried PLGA microspheres or swollen gelatin microspheres (560 µl dd$H_2O$/100 mg dry spheres) were mixed with the calcium phosphate cement powder before liquid hardener (1% $Na_2HPO_4$) was added. Alternatively, PTMC microspheres were dispersed into the liquid hardener before adding the cement powder[14]. The resulting mixtures were stirred vigorously for 15 s using a Silamat® mixing device (Vivadent, Schaan, Liechtenstein) and injected into Teflon molds with cylindrical holes (4.5 mm diameter, 9.0 mm length) and hardened at room temperature over night.

Properties of the resulting calcium phosphate cement composites with PLGA microspheres (PLGA CPC), gelatin type A microspheres (GELA CPC), gelatin type B microspheres (GELB CPC), LMW PTMC microspheres (LMW PTMC CPC) and HMW PTMC microspheres (HMW PTMC CPC) are summarized in Table 2[12,14,15]. In addition, a composite material was prepared which contained both PLGA and gelatin type A microspheres (PLGA/GELA CPC). To compare the *in vivo* response and degradation for all composites, macroporosity[4] (volume percentage of microspheres) was kept in the range of 48-57 %.

**Table 2.** Properties microsphere CPCs[12,14,15]

| Name | PLGA CPC | GELA CPC | GELB CPC | GELA/ PLGA CPC | LMW PTMC CPC | HMW PTMC CPC |
|---|---|---|---|---|---|---|
| Average size microspheres (μm) | 33 ± 17 | 37 ± 31 | 21 ± 15 | 33 ± 17, 37 ± 31 | 29 ± 17 | 15 ± 6 |
| Wt % microspheres | 20 % | 10 % | 5 % | 5 + 10 % | 25 % | 25 % |
| Vol % microspheres | 48 % | 57 % | 48 % | 53 % | 49 % | 49 % |

*2.2.5. Surgical procedure and implant retrieval*

Eighteen 6-month old female New Zealand white rabbits of 3-4 kg were used in this experiment. Surgery was performed under total anesthesia. Before surgery, the back of the rabbits was shaven, washed with alcohol and disinfected with iodine. Subsequently, at both sides of the vertical column, three 1.5 cm long incisions were made through the full thickness of the skin. Lateral pockets for subcutaneous implantation were made by blunt dissection with scissors. One implant of each formulation was inserted into each subcutaneous pocket, after which the pockets were closed with Vicryl 4.0 sutures. After 4, 8 and 12 weeks, 6 rabbits were sacrificed by $CO_2$ suffocation. Three specimens of each material and time point were used for physicochemical characterization and histology. For physicochemical analysis, the tissue that surrounded the samples was removed. Then, the specimens were stored in water. For histological examination, samples including their surrounding tissues were fixated in a 4% formaldehyde solution for 1 week, dehydrated by a series of alcohol dilutions and embedded in methyl methacrylate. After polymerization, undecalcified 10μm thick sections of the implant were prepared by a modified sawing microtome technique[22]. Three sections per implant were made and stained by methylene blue/basic fuchsin.

*2.2.6. Physicochemical analysis*

Mechanical strength

After implant retrieval, wet samples were subjected to compression strength measurements. Therefore, they were placed in a testing bench (858 MiniBionixII®, MTS Corp., Eden Prairie, MN, USA) and compression strength along the vertical axis (height) of the specimens was measured at 0.5 mm/min crosshead speed. Samples before implantation (after 1 day setting) were used as reference.

Molecular weight

Gel permeation chromatography (GPC) was used to determine the molecular weight of the polymer microspheres at different implantation time points. For the PTMC CPCs, GPC analysis was difficult, therefore only PLGA polymer inside the PLGA CPC and PLGA/GELA CPC was measured with GPC. For analysis, samples were grinded and extracted with 3 ml of THF. The resulting suspension was filtered using 13 mm GHP 0.2μm Acrodisk® filters (Waters). The HPLC system consists of a L2130 HPLC pump, a L-2400 UV detector and a L-2490 RI Detector (Hitachi corp., Tokyo, JPN). The GPC

column was a 4.6*300 mm Styragel® HR 4E column with a 4.6*30 mm Styragel® guard column (Waters Corp., Milford, MA, USA). Tetrahydrofuran (THF) was used as eluent, flow rate was 0.35 ml/min. To obtain a calibration curve, polystyrene standards (SM-105, Shodex corp., Tokyo, JPN) were used. For the calculation of the number and weight average molecular weight ($M_n/M_w$) of the PLGA, Mark-Houwink constants of PLGA 50:50 (a = 0.761, K = $1.07*10^{-2}$, THF, 22.5°C)[23] and PS (a = 0.717, K = $1.17*10^{-2}$, THF, 25°C) were used.

Infrared spectroscopy

For the PTMC CPCs, a fraction of the samples was grinded and measured by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR, Perkin Elmer, Fremont, CA, USA). A method derived from Featherstone et al[24] was used to quantitatively determine the content of PTMC in the composites in time and was also applied in a previous experiment[14]. Briefly, the extinction of a characteristic PTMC peak (C=O, 1750 $cm^{-1}$) and calcium phosphate peak ($PO_4$, 1050 $cm^{-1}$) was determined using Equation 1. According to this method, the ratio of both extinctions ($E_{C=O}/E_{PO4}$) forms a linear relationship with the percentage of PTMC within the composites. For this, a standard curve was made using samples with different PTMC/cement compositions and calibrated with thermogravimetric analysis (TGA). This resulted in a linear relationship for both low and high molecular weight polymers ($r^2 > 0.98$), which was used to determine the amount of PTMC in the samples.

$$E = \log \frac{T_{baseline}}{T_{sample\_peak}} \qquad \textit{Equation 1}$$

*Legend:* E = extinction, $T_{baseline}$ = transmission baseline, $T_{sample\ peak}$ = transmission sample peak

X-ray diffraction

The calcium phosphate cement composition of the microsphere/CPC formulations was determined by powder X-ray diffraction (XRD, Philips, PW 3710, Almelo, The Netherlands). The same grinded samples were used as for FTIR.

SEM

The morphology of the different microsphere/CPC formulations was investigated by scanning electron microscopy (SEM, JEOL 6400-LINK AN 10000 at 10 kV). Samples were dried on carbon tape and sputtered with gold prior to measurement. Magnifications of 250/500x were used to visualize microsphere degradation.

*2.2.7. Histological examination*

After sectioning of the histological samples, tissue response and microsphere degradation was examined by light microscopy (Leica Microsystems AG, Wetzlar, Germany). A histological grading scale for soft-tissue implants[25] was used to evaluate the tissue response surrounding the implant

(reaction zone) and at the implant surface (interface) (Table 3). Two observers conducted the scoring separately and reached consensus on the final score.

**Table 3.** Histological grading scale of the tissue reaction[25]

| Evaluation | Response | Score |
|---|---|---|
| Capsule Quantitatively | 1-4 fibroblasts | 4 |
| | 5-9 fibroblasts | 3 |
| | 10-30 fibroblasts | 2 |
| | > 30 fibroblasts | 1 |
| Capsule Qualitatively | Capsule is fibrous, mature, not dense, resembling connective or fat tissue in the non-injured regions | 4 |
| | Capsule tissue is fibrous but immature, showing fibroblasts and little collagen | 3 |
| | Capsule tissue is granulous and dense, containing both fibroblasts and many inflammatory cells | 2 |
| | Capsule consists of masses of inflammatory cells with little or no signs of connective tissue organization | 1 |
| Interface Qualitatively | Fibroblasts contact the implant surface without the presence of macrophages or leucocytes | 4 |
| | Scattered foci of macrophages and leucocytes are present | 3 |
| | One layer of macrophages and leucocytes are present | 2 |
| | Multiple layers of macrophages and leucocytes are present | 1 |

*2.2.8. Statistical analysis*

Data were arranged as mean ± standard deviation. Significant differences were determined using analysis of variance (ANOVA). Results were considered significant if $p<0.05$. Calculations were performed using GraphPad Instat® (GraphPad Software Inc., San Diego, Ca, USA).

## 3. Results

### *3.1. Implant retrieval*

During the experiment one rabbit died as a result of a bacterial infection. Hence, of the 8 weeks group one implant of each formulation was lost. The other seventeen rabbits remained in a good health and did not show any wound complications. At the end of the implantation periods, a total of one hundred and two implants (17 of each formulation) were retrieved. At retrieval, the implants were all covered by an intact fibrous tissue capsule.

### *3.2. Physicochemical analysis*

Results of the compression strength measurements after 4, 8 and 12 weeks of implantation are depicted in Figure 1. In most cases compression strength decreased gradually during implantation time. For the PLGA CPC, PLGA/GELA CPC and HMW PTMC CPC, compression strength was significantly decreased after 12 weeks of implantation. For the GELB CPC compression strength significantly decreased from 11MPa initially to 4 MPa at 8 weeks of implantation, which was followed by a small increase in compression strength at 12 weeks.

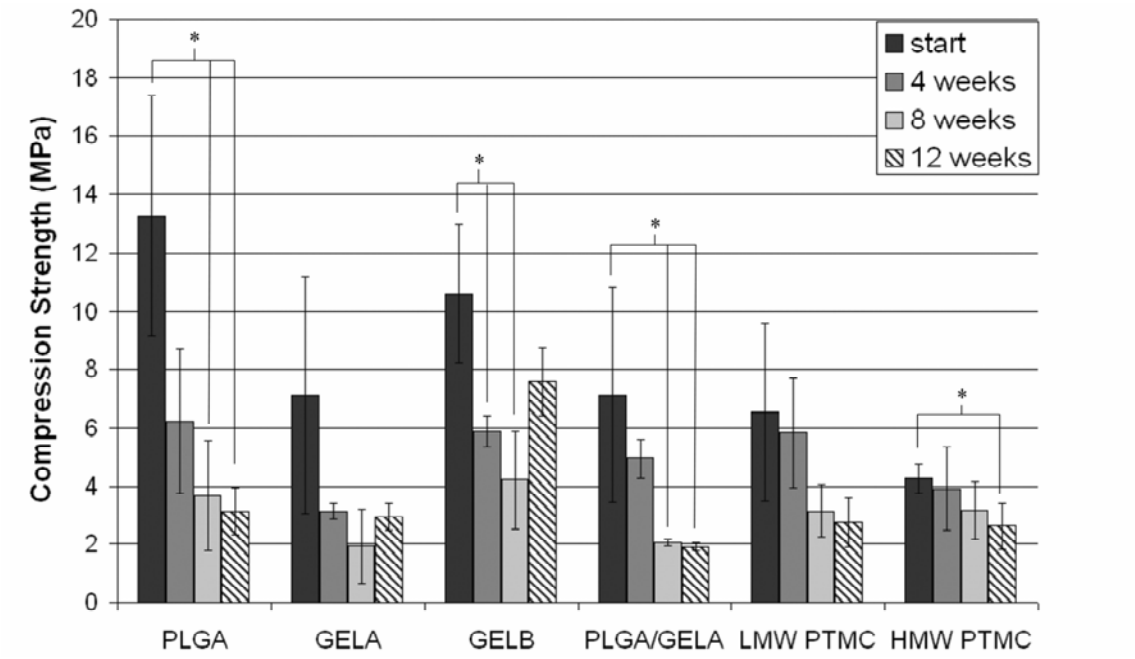



**Figure 1.** Compression strength of samples of different microsphere/calcium phosphate cement formulations as a function of implantation period

Results of gel permeation chromatography (Figure 2) show that the number average molecular weight ($M_n$) of the PLGA polymer inside the PLGA CPC and PLGA/GELA CPC gradually decreased to ~ 5000 g/mol at 8 weeks of implantation. At 12 weeks not enough PLGA polymer was left for GPC analysis.

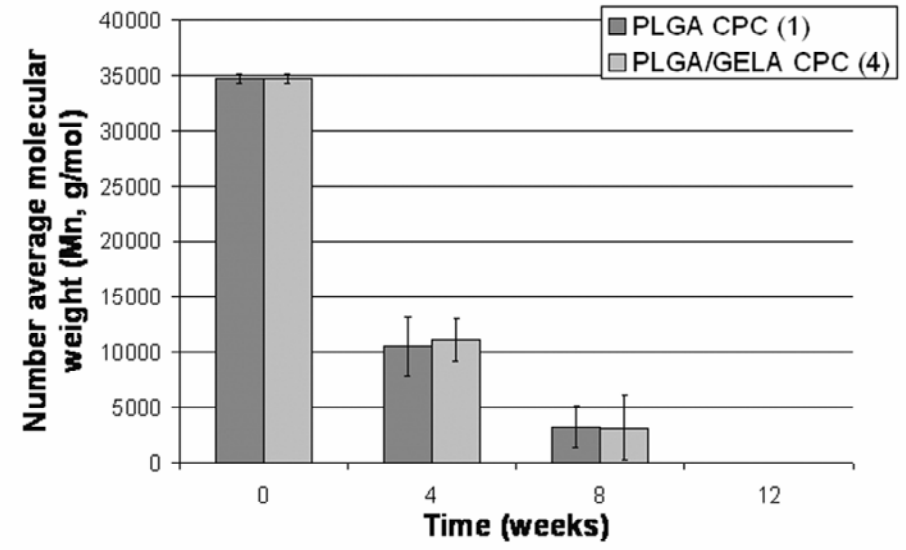



**Figure 2.** Number average molecular weight of PLGA of the PLGA CPC and PLGA/GELA CPC as a function of implantation period

ATR-FTIR (Figure 3) revealed that the PTMC CPCs exhibited microsphere degradation after 4-12 weeks as the percentage of PTMC inside the composites significantly decreased to ~ 70% of its original value. Between the low and high molecular weight PTMC, no significant differences in degradation rate were observed. The spectrograms also indicated that after subcutaneous implantation an additional peak appeared at 1650 $cm^{-1}$ corresponding to the C=O stretch of proteins[26].

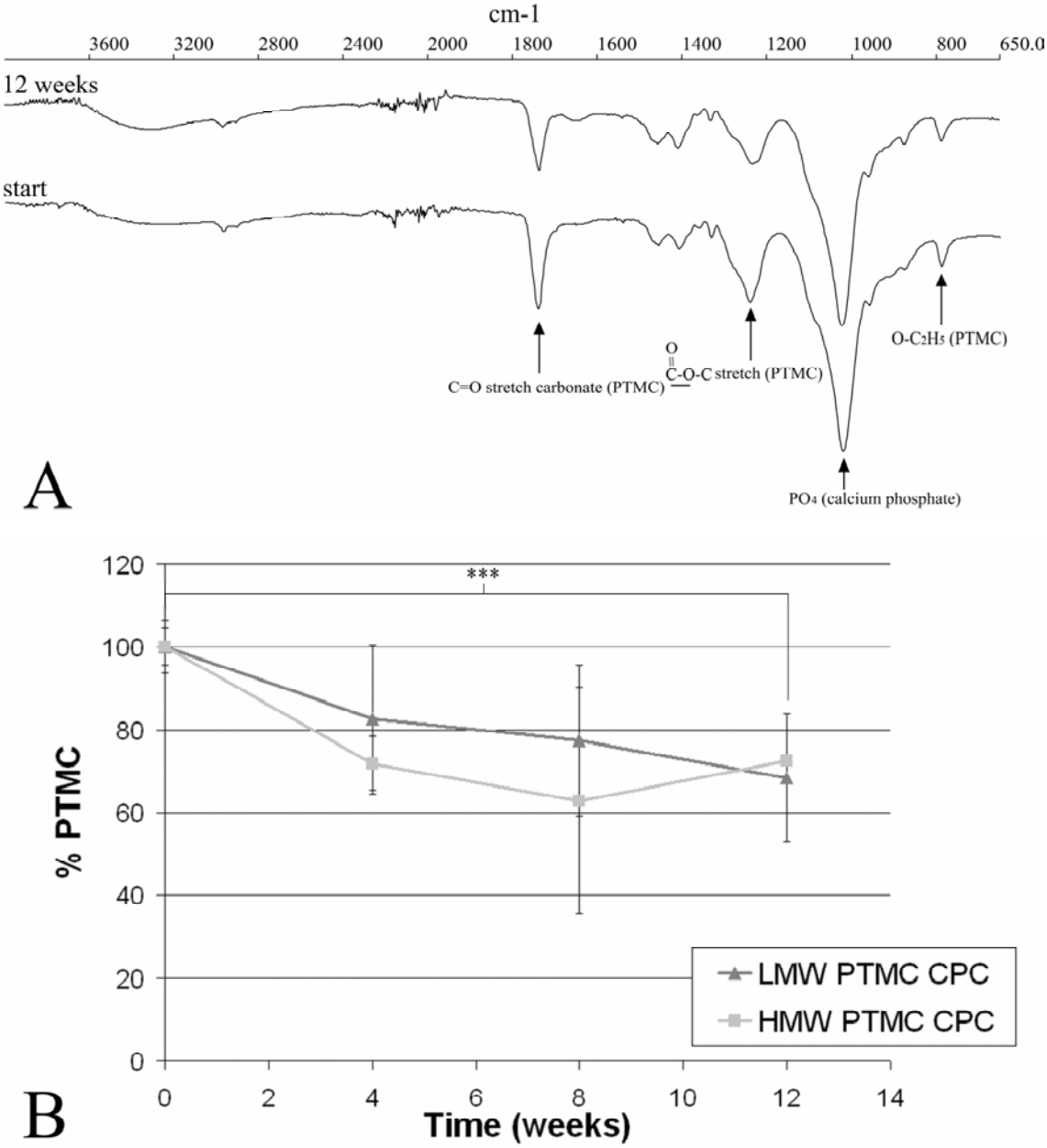



**Figure 3.** Determination of the amount of PTMC in the PTMC CPCs; A. IR spectra HMW PTMC CPC of starting material and at t=12 weeks, B. amount of PTMC as % of initial amount of PTMC in both LMW PTMC CPC and HMW PTMC CPC scaffolds as a function of implantation period, *** = $p<0.005$

In the SEM-micrographs (Figures 4-5), degradation of the PLGA was observed for both PLGA CPC and PLGA/GELA CPC samples as characterized by a deformation of the microspheres at 4 weeks and complete disappearance of the microspheres at 8 and 12 weeks of implantation.

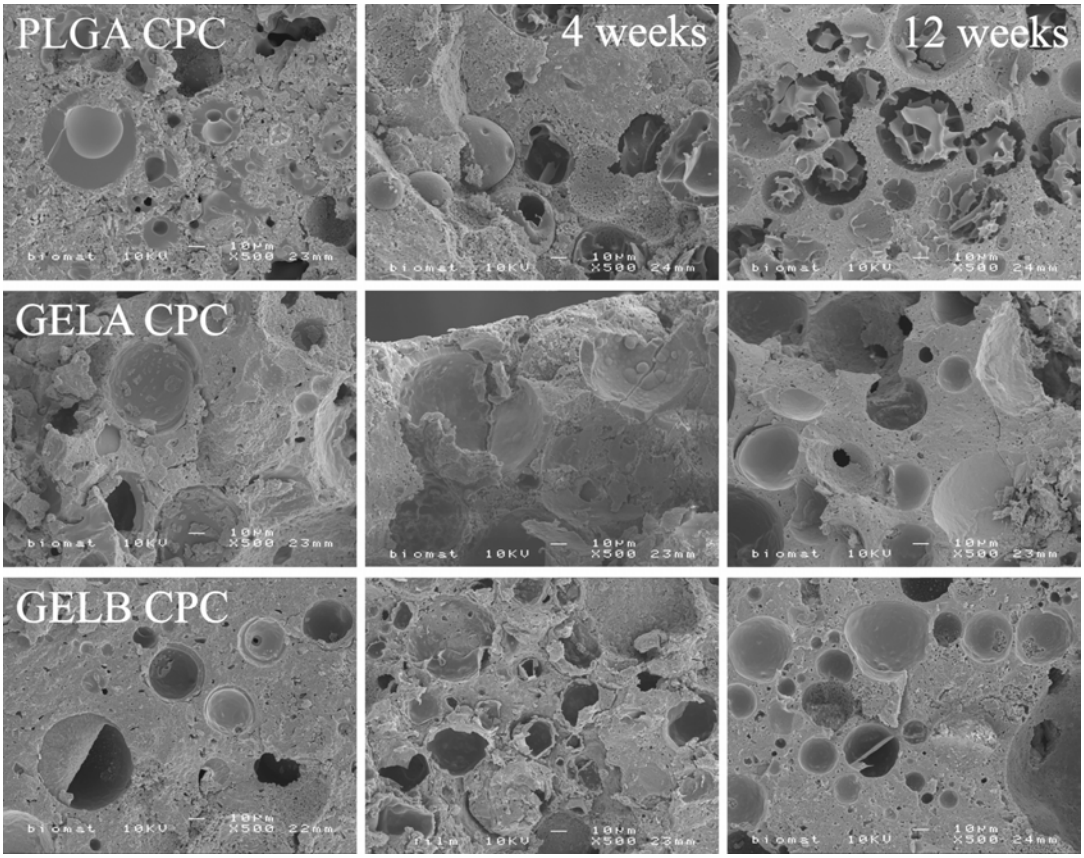



**Figure 4.** SEM-micrographs of PLGA CPC, GELA CPC and GELB CPC samples at t = 0, 4 and 12 weeks of implantation (original magnification 500x)

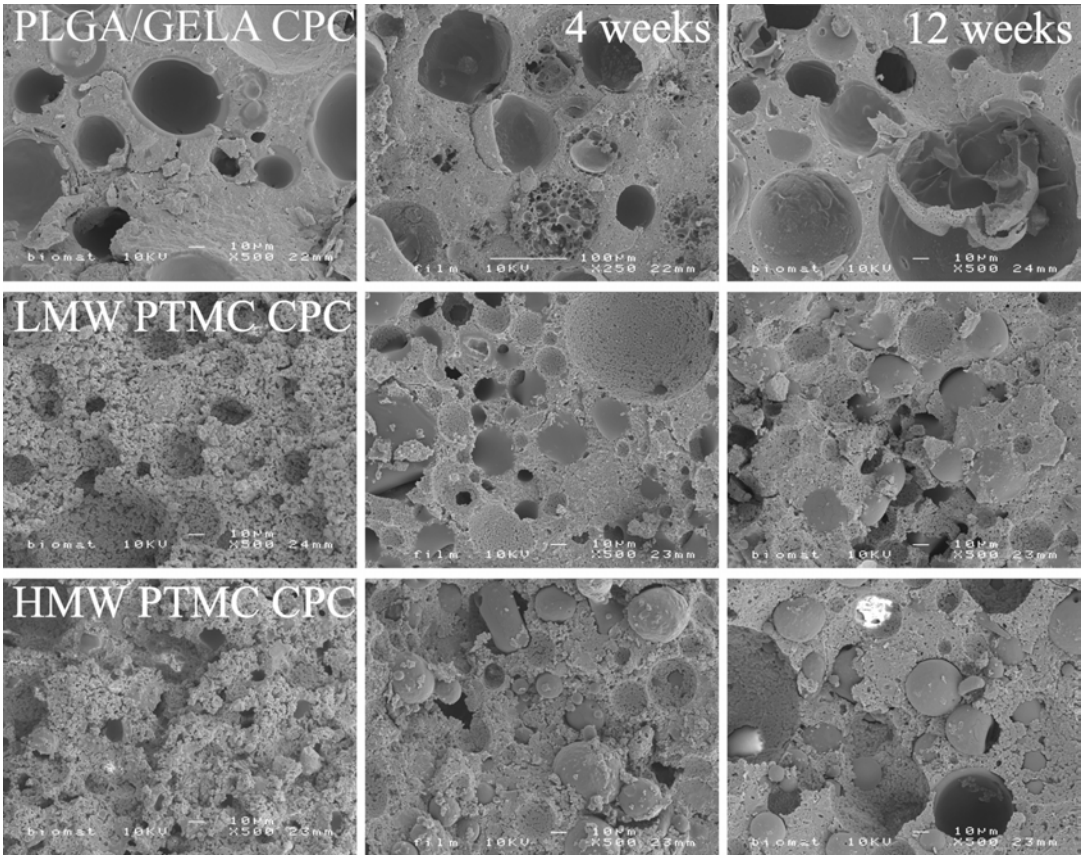



**Figure 5.** SEM-micrographs of PLGA/GELA CPC, LMW PTMC CPC and HMW PTMC CPC after t = 0, 4 and 12 weeks of implantation (original magnifications 250 and 500x

In the GELA CPC, GELB CPC and PLGA/GELA CPC, gelatin shells were still visible at 12 weeks of implantation. In time, the morphology of the gelatin microspheres changed. Initially, the microspheres had a thick shell[13, 15], but at 8 and 12 weeks the thickness decreased slowly, which was also associated with deformations of the shell wall. The occurrence of gelatin degradation was further confirmed by the observation that at 12 weeks the composites showed a distinctive higher porosity than after 4 weeks (Figure 6). In all gelatin microsphere containing CPCs also some proof of tissue ingrowth was observed as is visualized in Figures 6C and 6D.

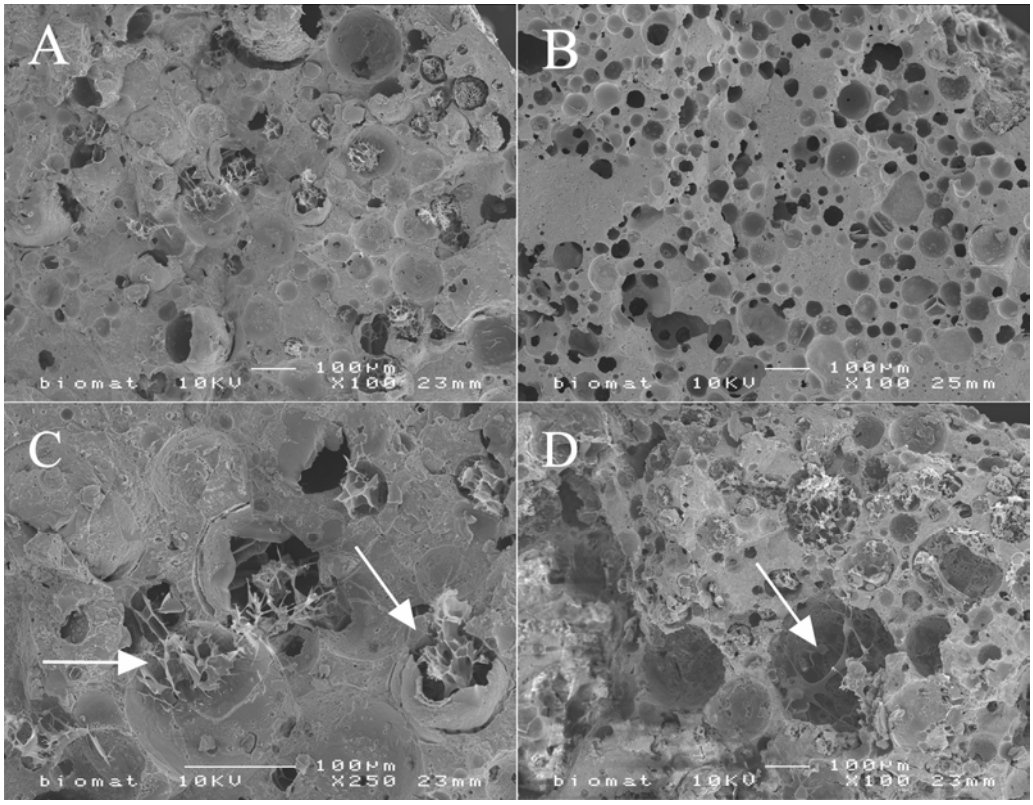



**Figure 6.** SEM micrographs of GELA CPC at t = 4 (A) and 12 weeks (B) of implantation, close-up picture GELA CPC at t = 4 weeks of implantation (C) and SEM micrograph of PLGA/GELA CPC at t = 8 weeks of implantation (D) (arrows denote tissue ingrowth, original magnifications 100x and 250x)

The PTMC CPCs showed the presence of microsphere structures at all time points. However, microspheres were deformed plastically in LMW PTMC CPC. In the HMW PTMC CPC, the microspheres maintained a spherical structure although the diameter of the spheres decreased during implantation resulting in an empty space between the microsphere and the surrounding spherical hole. Furthermore, it was observed that in HMW PTMC CPC, spheres were often fused together (Figure 7). At a fracture plane, HMW PTMC microspheres were found to have become interconnected, while in the LMW PTMC CPC the polymer showed elongation without the retention of microsphere structure.

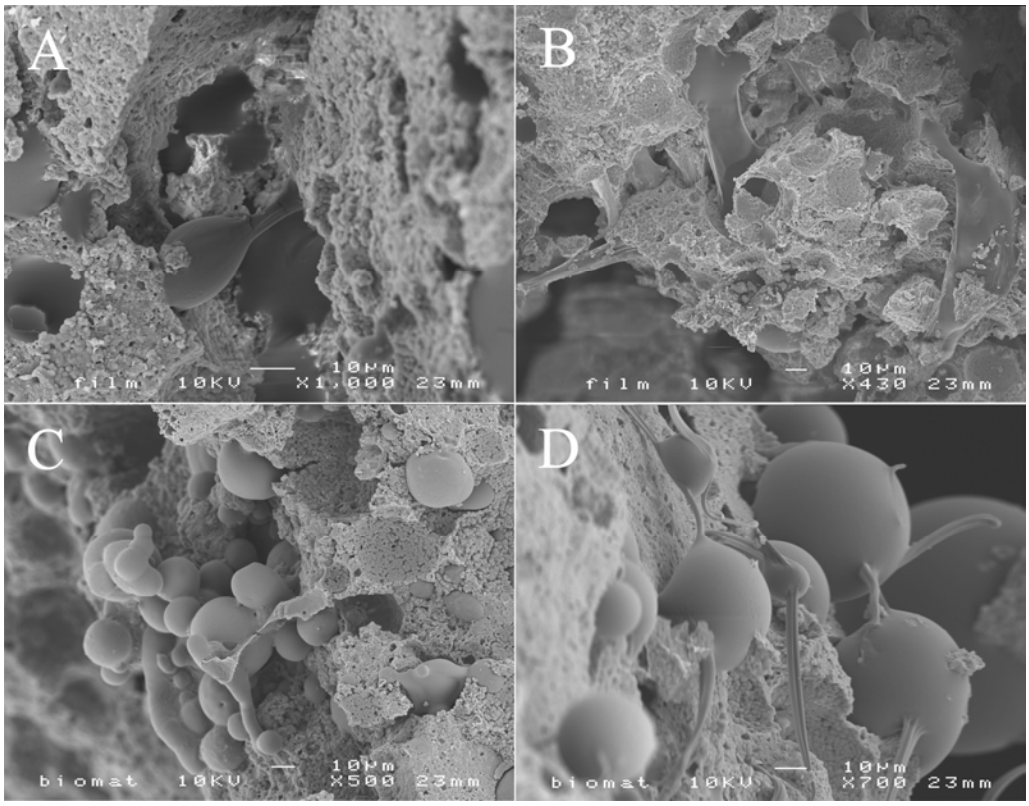



**Figure 7.** SEM micrographs LMW PTMC CPC at t=4 weeks of implantation indicating stretching of the material at a fracture plane (A + B, original magn. 1000 and 430x), agglomeration of HMW PTMC microspheres (C, original magn 500x) and agglomerated HMW PTMC microspheres at fracture interface (D, original magn 700x)

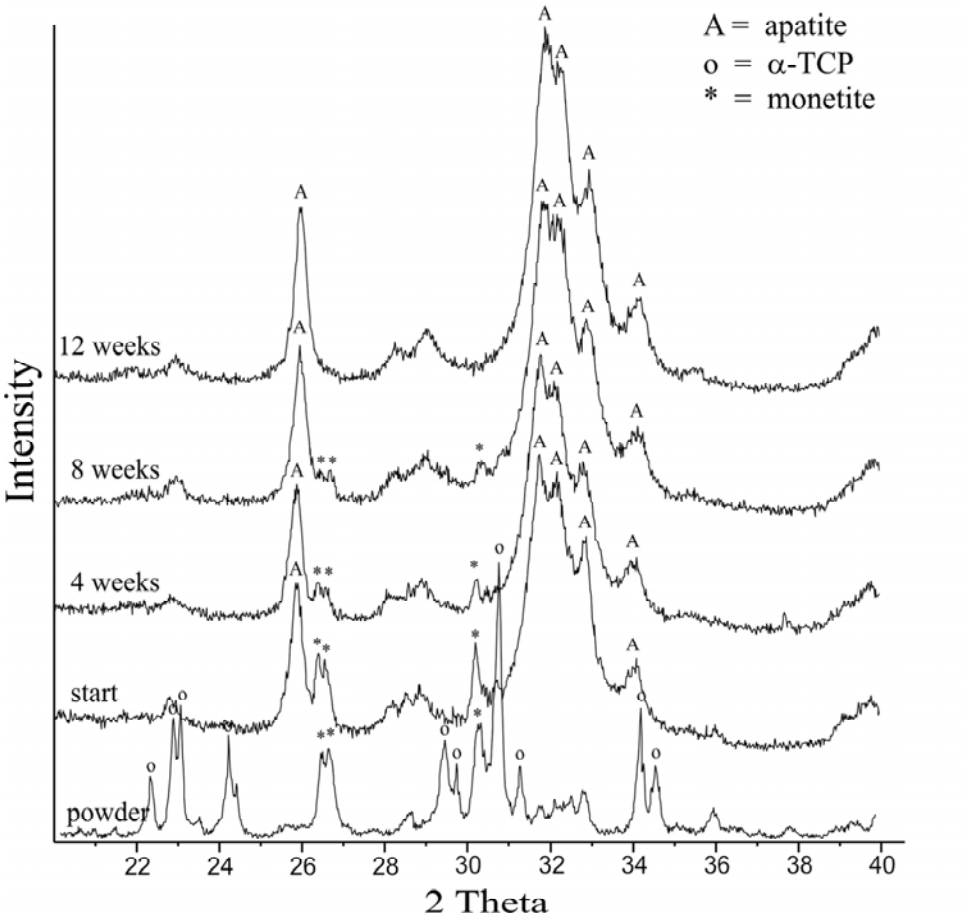



**Figure 8.** XRD-spectra of GELB CPC featuring cement powder, starting material (t=0) and samples after 4, 8 and 12 weeks of implantation

Finally, investigation of the cement composition with XRD showed that for all microsphere CPCs, monetite and α-TCP signals completely disappeared within the 12 week implantation period implying that the cement has been transformed into apatite. Typical XRD-spectra were shown in Figure 8.

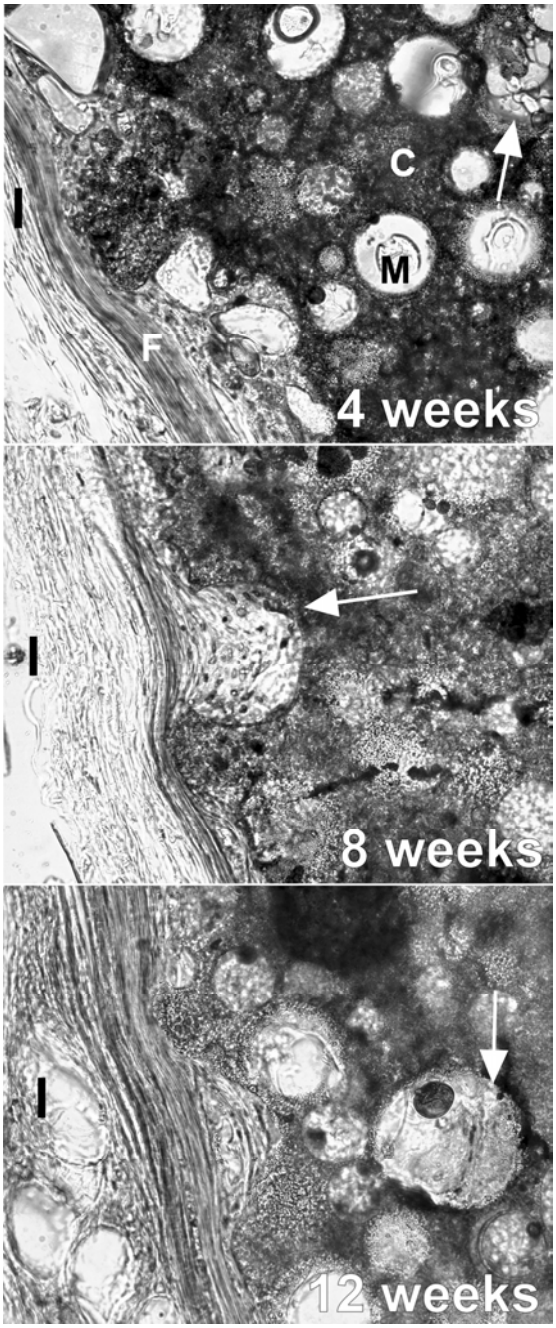



**Figure 9A.** Histological sections PLGA CPCs at 4, 8 and 12 weeks of subcutaneous implantation, C = cement, M = microsphere, F = fibrous capsule, arrows denote tissue ingrowth in microspheres (bar represents 25μm). *See also page 198 for color figure.*

### *3.3. Histological examination*

Light microscopical pictures of the various histological specimens are depicted in Figures 9A-B. After 4 weeks of implantation, the surface of the implants showed a rough appearance with spherical cavities corresponding to degraded microspheres and irregular shaped calcium phosphate particles. Tissue response to all microsphere CPC/formulations was similar, and revealed a fibrous capsule of typically > 10 layers that was composed of aligned fibroblasts and other cells. In more than 50% of the samples,

this fibrous capsule contained some inflammatory cells. Occasionally, between the fibrous capsule and cement surface multiple layers of inflammatory cells were observed, which could be associated with the presence of multi-nucleated cells at the cement surface. At places where the implant showed a smooth surface, little or no inflammatory response was seen. For the GELA CPC, GELB CPC and GELA/PLGA CPC, cells were seen in the microsphere cavities till a distance of >100μm below the implant surface.

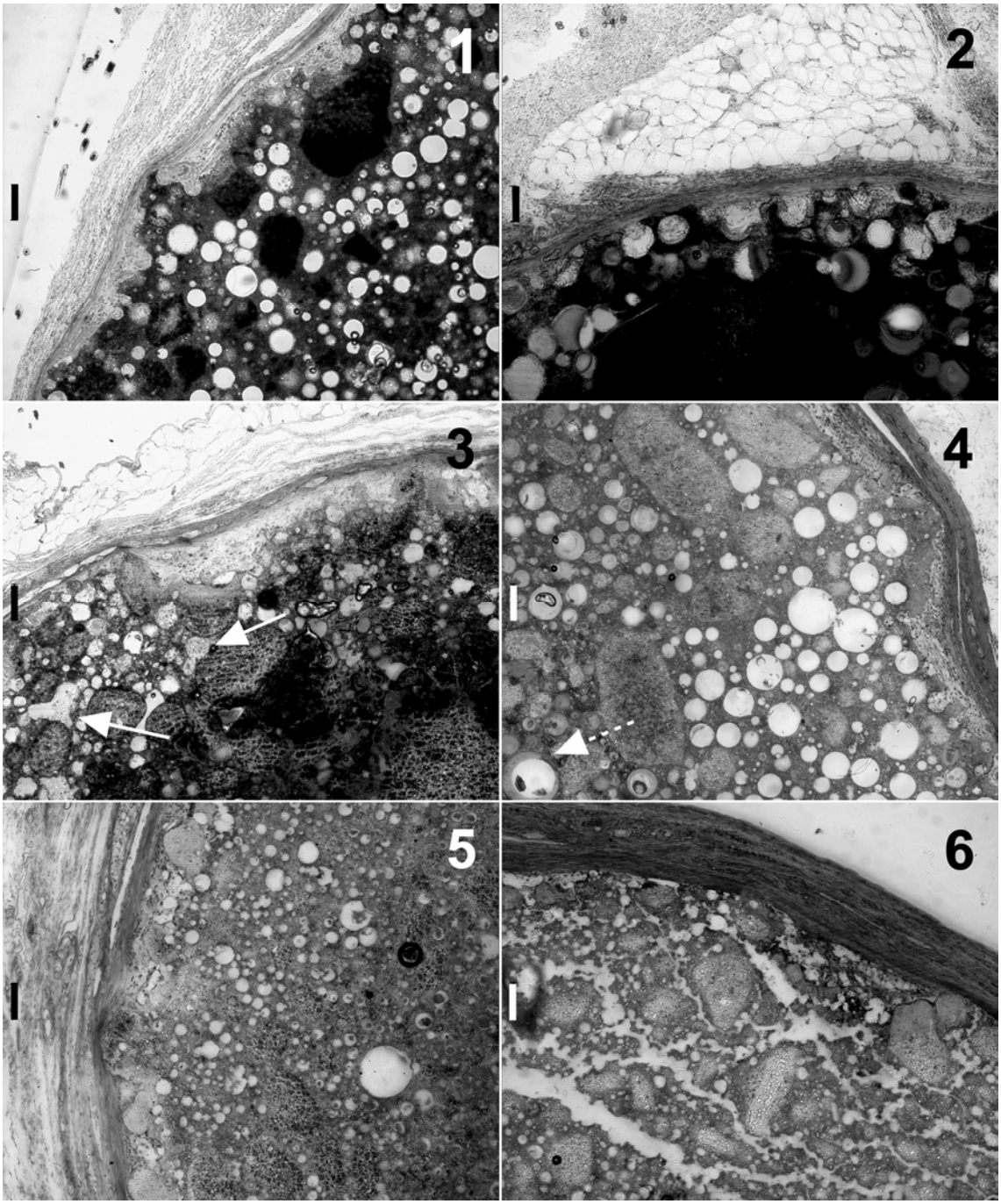



**Figure 9B.** Histological sections of microsphere CPCs after 12 weeks of implantation; PLGA CPC (1), GELA CPC (2), GELB CPC (3), PLGA/GELA CPC (4), LMW PTMC CPC (5) and HMW PTMC CPC (6), arrows denote tissue ingrowth with GELB CPC, dashed arrow denotes shell gelatin microsphere (bar represents 100 μm). *See also page 199 for color figure.*

The 8 and 12 week specimens showed a gradual decrease in fibrous capsule thickness, especially for PLGA CPC and GELB CPC. Tissue ingrowth into the various microsphere/CPC formulations increased during implantation time. Tissue response at these time points was similar to the 4 weeks samples.

Besides differences in tissue response, some distinct differences regarding structure or microsphere degradation were visible with light microscopy. At t = 4 weeks all gelatin microsphere CPCs looked similar and microspheres were evenly spread throughout the material. Gelatin microspheres could be easily distinguished from the surrounding cement or pores because they adsorbed high quantities of dye. Morphology did not change for GELA CPC after t = 8 and 12 weeks, while for the PLGA/GELA CPC and GELB CPC the microspheres at the outer parts of the samples slowly degraded in time. Furthermore, for the HMW PTMC CPC, the cement at the outer parts was fractured at all time points, while for the other microsphere/CPC formulations the cement structure was intact.

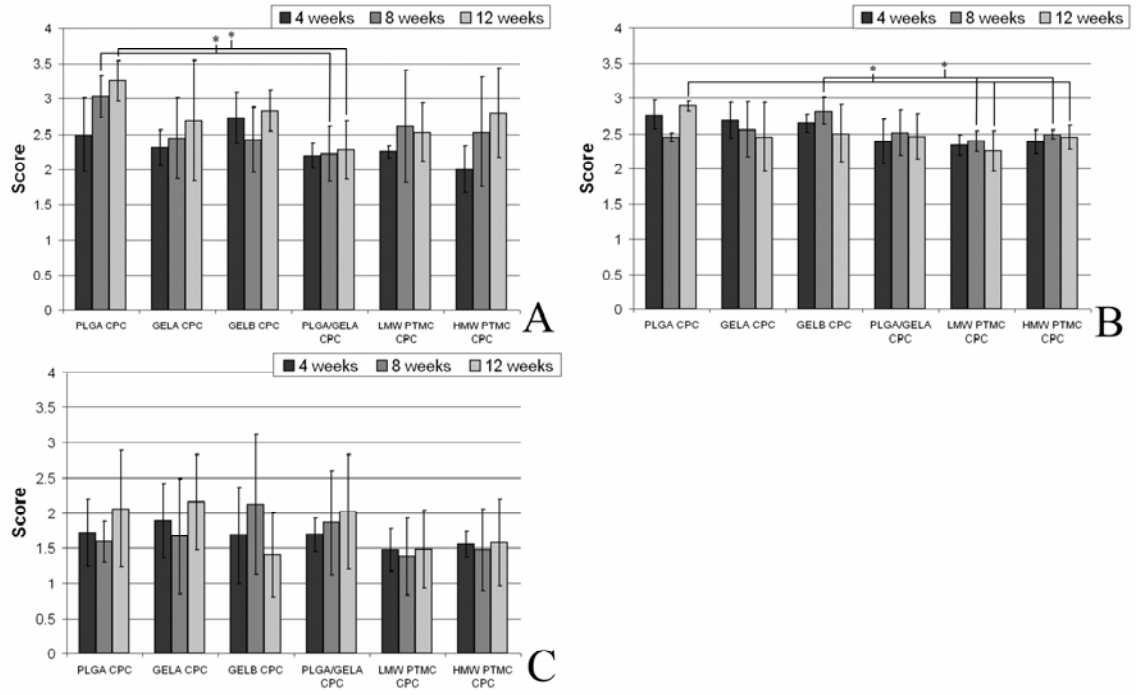



**Figure 10.** Histological scoring of samples after 4, 8 and 12 weeks of implantation; A. Capsule quantitatively, B. Capsule qualitatively, C. Interface qualitatively; * = $p<0.05$

Histological grading results are given in Figure 10. For all microsphere CPCs, fibrous capsule thickness scored between 2-2.5 at week 4 and appeared to show a gradual decrease over time. Significant differences in capsule thickness were found between the PLGA CPC and PLGA/GELA CPC after 8 and 12 weeks ($p<0.05$). The fibrous capsule quality was similar for most microsphere/CPC formulations and time points (an average scoring of 2.5), but for the GELB CPC and PLGA CPC a statistically slightly higher capsule quality ($p<0.05$) was obtained compared to both PTMC CPCs after 8 and 12 weeks respectively. The implant interface quality data showed an average

score of 1.5-2.0 for all groups and time points, which did not change over time. Further, the interface data did not show significant differences between the various microsphere/CPC formulations.

## 4. Discussion

In this study, composites of calcium phosphate cements with incorporated PLGA, gelatin or PTMC microspheres were implanted subcutaneously into the back of rabbits to investigate the *in vivo* degradation characteristics and to evaluate the biocompatibility of these materials. In *in vitro* conditions, macroporosity was generated by various microsphere degradation and erosion mechanisms after incubation of PLGA CPC in phosphate buffered saline (PBS)[12], or incubation of gelatin CPC and PTMC CPC in enzyme containing media[14,15]. Although in an *in vivo* environment gelatin and PTMC degrading enzymes are present, degradation patterns can be different due to an altered fluid flow, buffer capacity and enzyme activity.

Results from GPC analysis show that the molecular weight ($M_n$ and $M_w$) of PLGA microspheres within the composites decreased continuously from the beginning, and were substantially degraded within 12 weeks of implantation. This degradation pattern was confirmed by compression strength data that showed a continuous and significant decrease over time. Also the SEM micrographs confirmed the complete disappearance of the microspheres after 8 and 12 weeks, whereas at 4 weeks microspheres are still present. These results correspond to an earlier performed *in vitro* degradation study[12] where microspheres prepared from PLGA with a similar molecular weight were applied. Evidently, the degradation mechanism of PLGA (hydrolysis, bulk erosion) is also valid in an *in vivo* environment.

For gelatin microsphere CPCs it is difficult to investigate the microsphere degradation because both GPC and FTIR cannot be applied. Although water-phase GPC is available to investigate the molecular weight of proteins, gelatin consists of a wide range of different molecular weights and does not give a clear Gaussian distribution. Holland et al[19] used the microsphere swelling characteristics to determine hydrogel degradation, but in the gelatin CPCs microspheres are trapped inside a stiff calcium phosphate matrix. In addition, FTIR cannot be used to investigate *in vivo* microsphere degradation as gelatin does not give sharp, distinctive peaks and the percentage (5-10wt%) of microspheres is small compared to the cement phase. Furthermore, additional proteins that are absorbed by the cement will give a signal at the same place (1650 $cm^{-1}$) as gelatin, as was also visible in the FTIR-curves of HMW PTMC CPC.

Despite the fact that it is difficult to quantify gelatin microsphere degradation inside these composites, the gelatin microspheres do show degradation as next to the decrease in compression strength, a distinct increase in porosity of the composite was observed in SEM. Histological examination provided a better indication of the degradation mechanism for both GELB CPCs and PLGA/GELA CPCs. It was observed that microsphere degradation proceeded from the outside to the center of the material, which corresponds to the *in vitro* degradation behavior[15]. It has to be noticed that GELA

CPC did not show this degradation pattern, as probably caused by the overall thicker shell of these microspheres, which is more difficult to degrade completely. Apparently, the combination of GELA and PLGA microspheres enhances the GELA microsphere degradation. This can be due to the higher porosity as generated after PLGA microsphere degradation that facilitates enzyme diffusion into the composite.

Histological evaluation and investigation by SEM confirmed the ingrowth of cells at a distance of >100 μm into the GELA CPC, GELB CPC and PLGA/GELA CPC composites, which indicates that the porosity generated by the microspheres was interconnected[27]. Nevertheless, cell ingrowth never reached the center of the implant as was observed by Link et al[28] with PLGA CPC. A too small size of the PLGA microspheres[28] or the formation of calcium phosphate precipitate in or onto the gelatin microspheres[13, 15] could have hampered connectivity of the pores in our study and hindered tissue ingrowth.

Analysis with FTIR revealed that both LMW PTMC CPCs and HMW PTMC CPCs showed microsphere degradation as the percentage of PTMC inside the composites significantly decreased upon subcutaneous implantation. Furthermore, SEM-investigation showed that the HMW PTMC microspheres became smaller than the surrounding spherical hole, which indicates the occurrence of surface erosion of the microspheres.

No significant differences in degradation rate between the LMW PTMC and HMW PTMC were observed and both formulations exhibited about 30% degradation at 12 weeks of implantation. Alternatively, studies by Zhang et al[16] where PTMC disks were implanted in the femur and tibia of rabbits, have shown that the highest molecular weight PTMC exhibits a faster *in vivo* degradation as the enzymes (lipases) degrade hydrophobic polymers, i.e. ones with higher molecular weights, faster than the lower molecular weight polymers. In a previous *in vitro* degradation experiment with PTMC CPCs in lipase solution[14], the highest molecular weight polymer microspheres ($PTMC_{176}$, Mn = 176 kDa) degraded even slower than the lower molecular weight microspheres ($PTMC_{53}$, Mn = 53 kDa). The explanation for this observation was that the presence of a hydrophilic layer of poly(vinyl alcohol) (PVA) at the surface of the microspheres delayed PTMC degradation of the smaller HMW PTMC microspheres. In our study, the difference in molecular weight between the low and high molecular weight PTMC polymer (40 kDa to 444 kDa) was larger than in the previous experiment. Alternatively, also different environments or enzyme sources could have influenced the *in vivo* degradation of these PTMC CPC composites.

In agreement with our previous *in vitro* degradation experiment, elongation of PTMC microspheres at a fracture surface was observed in SEM-micrographs[14]. For the HMW PTMC CPCs, also agglomeration of the microspheres was observed. This is beneficial for tissue ingrowth as interconnections between pores will be present after microsphere degradation. Cracks that were observed in the HMW PTMC histology samples after incubation were due to swelling of the polymer in acetone and methyl methacrylate.

Finally, the histological grading data revealed that especially the implant interface of all microsphere/cement formulations showed a moderate inflammatory response as inflammatory cells were present between the fibrous tissue capsule and cement surface. This was in contrast to previous studies where CPC[29] or PLGA CPC[28] was subcutaneously implanted in goats as well as rats and only a limited inflammatory response was observed. The thickness of the fibrous capsule, however, was similar to these studies. An explanation for the higher inflammatory response at the implant interface can be found in the irregular shaped surface of the microsphere/calcium phosphate cement composites[29] that was caused by microsphere degradation. Also the animal model can influence the inflammatory response as the morphology of the skin and subcutaneous tissue of the rabbit are different compared with the rat and goat. Furthermore as all composites showed a similar tissue response, likely degradation products of the microspheres like lactic acid, or remaining gelatin[30] or glutaraldehyde[31] did not contribute to the inflammatory response.

## 5. Conclusion

Composites composed of calcium phosphate cement with incorporated PLGA, gelatin or PTMC microspheres showed *in vivo* degradation of the microspheres with corresponding decrease in mechanical strength. PLGA microspheres exhibited bulk erosion throughout the composites, whereas with gelatin type B degradation of the microspheres started at the outside and gradually proceeded to the inner part. Furthermore, with HMW PTMC, surface erosion of microspheres over time was observed. Histological analysis revealed that for all composites a similar tissue response was observed with a decreasing capsule thickness over time and a moderate inflammatory response between the fibrous capsule and cement surface. Overall, microsphere CPCs can be used to generate porous scaffolds in an *in vivo* environment after degradation of microspheres with various degradation/erosion mechanisms.

*Acknowlegdements*

Authors would like to thank Natasja van Dijk and Martijn Martens for the histological preparations and Vincent Cuijpers for technical assistance. The authors would also like to thank dr. Frank Walboomers for his help with histological scoring. Scanning electron microscopy was performed at the Microscopic Imaging Centre (MIC) of the Nijmegen Centre for Molecular Life Sciences (NCMLS), the Netherlands. Supported by the Dutch Technology Foundation STW, grant # NGT 6205.

## 6. References

1) E.M. Ooms, J.G.C. Wolke, J.P. van der Waerden, J.A. Jansen, Trabecular bone response to injectable calcium phosphate (Ca-P) cement, J Biomed Mater Res Part A. 61(1) (2002) 9-18

2) F. Theiss, D. Apelt, B. Brand, A. Kutter, K. Zlinsky, M. Bohner, S. Matter, C. Frei, J.A. Auer, B. von Rechenberg, Biocompatibility and resorption of a brushite calcium phosphate cement, Biomaterials. 26(21) (2005) 4383-4394

3) J.M. Kuemmerle, A. Oberle, C. Oechslin, M. Bohner, C. Frei, I. Boecken, B. von Rechenberg, Assessment of the suitability of a new brushite calcium phosphate cement for cranioplasty – an experimental study in sheep, J Craniomaxillofac Surg. 33(1) (2005) 37-44

4) Habraken WJEM, Wolke JGC, Jansen JA. Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. Adv Drug Deliv Rev 59(4-5) 2007 234

5) R.P. Del Real, E.M. Ooms, J.G.C. Wolke, M. Vallet-Regí, J.A. Jansen, In vivo bone response to porous calcium phosphate cement, J Biomed Mater Res Part A. 65(1) (2003) 30-36

6) R.P. Del Real, J.G.C. Wolke, M. Vallet-Regí, J.A. Jansen, A new method to produce macropores in calcium phosphate cements, Biomaterials. 23(17) (2002) 3673-3680

7) M.P. Ginebra, J.A. Delgado, I. Harr, A. Almirall, S. Del Valle, J.A. Planell, Factors affecting the structure and properties of an injectable self-setting calcium phosphate foam, J Biomed Mater Res Part A. 80(2) (2007) 351-361

8) H.H. Xu, M.D. Weir, E.F. Burguera, A.M. Fraser, Injectable and macroporous calcium phosphate cement scaffold, Biomaterials. 27(24) (2006) 4279-4287

9) C.G. Simon Jr, C.A. Khatri, S.A. Wight, F.W. Wang, Preliminary report on the biocompatibility of a moldable, resorbable, composite bone graft consisting of calcium phosphate cement and poly(lactide-co-glycolide) microspheres, J Orthop Res. 20(3) (2002) 473-482

10) P.Q. Ruhé, E.L. Hedberg, N. Torio Padron, P.H.M. Spauwen, J.A. Jansen, A.G. Mikos AG, rhBMP-2 release from injectable poly(D,L-lactic-*co*-glycolic acid)/calcium phosphate cement composites, J Bone Joint Surg Am. 85A(3) (2003) 75-81

11) J. Schnieders, U. Gbureck, R. Thull, T. Kissel, Controlled release of gentamycin from calcium phosphate-poly(lactic acid-co-glycolic acid) composite bone cement, Biomaterials. 27(23) (2006) 4239-4249

12) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cemens: physical properties and degradation characteristics, J Biomat Sci Polym Ed. 17(9) (2006) 1057-1074

13) W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, A.G. Mikos, J.A. Jansen, Introduction of gelatin microspheres into an injectable calcium phosphate cement, J Biomed Mater Res Part A. Published online January 11, 2008

14) W.J.E.M. Habraken, Z. Zhang, J.G.C. Wolke, D.W. Grijpma, A.G. Mikos, J. Feijen, J.A. Jansen, Introduction of enzymatically degradable poly(trimethylene carbonate) microspheres into an injectable calcium phosphate cement, Biomaterials. Published online (2008)

15) W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Porcine gelatin microsphere/ calcium phosphate cement composites: *in vitro* degradation and drug release, *Submitted to J Biomed Mater Res Part B: Appl Biomat*

16) Z. Zhang, R. Kuijer, S.K. Bulstra, D.W. Grijpma, J. Feijen, The in vivo and in vitro behavior of poly(trimethylene carbonate), Biomaterials. 27(9) (2006) 1741-1748

17) T.A. Holland, A.G. Mikos, Advances in drug delivery for articular cartilage, J Control Release. 86(1) (2003) 1-14

18) L. Lu, G.N. Stamatas, A.G. Mikos, Controlled release of transforming growth factor β1 from biodegradable polymer microparticles, J Biomed Mater Res Part A. 50 (2000) 440-451

19) T.A. Holland, J.K. Tessmar, Y. Tabata, A.G. Mikos, Transforming growth factor-β1 release from oligo(poly(ethylene glycol) fumarate) hydrogels in conditions that model the cartilage wound healing environment, J Control Release. 94(1) (2004) 101-114

20) Z. Zhang, M.A. Foks, D.W. Grijpma, J. Feijen, PTMC and MPEG-PTMC microparticles for hydrophilic drug delivery, J Control Release. 101(1-3) (2005) 392-394

21) G. Schwach , N. Oudry, S. Delhomme, M. Luck, H. Lindner, R. Gurny, Biodegradable microparticles for sustained release of a new GnRH antagonist—part I: Screening commercial PLGA and formulation technologies, Eur J Pharm Biopharm. 56 (2003) 327

22) J.P. Klein, Y.M. Sauren, W.E. Modderman, J.P. van der Waerden, A new saw technique improves preparation of bone sections for light and electron microscopy, J Appl Biomater. 5 (1994) 369-373

23) K. Schwartz, M. Epple, A detailed characterization of polyglycolide prepared by solid-state polycondensation reaction, Macromol Chem Phys. 200(10) (1999) 2221-2229

24) J.D.B. Featherstone, S. Pearson, R.Z. LeGeros, An Infrared method for quantification of carbonate in carbonated apatites, Caries Res. 18 (1984) 63-66

25) J.A. Jansen, W.J. Dhert, J.P. van der Waerden, A.F. von Recum, Semi-quantitative and qualitative histologic analysis method for the evaluation of implant biocompatibility, J Invest Surg. 7 (1994) 123-134

26) C.J. Pouchert (Editor). The Aldrich library of infrared spectra Edition III. 1981 page 435

27) V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials. 26 (2005) 5474-5491

28) D.P. Link, J. van den Dolder, W.J. Jurgens, J.G. Wolke, J.A. Jansen, Mechanical evaluation of implanted calcium phosphate cement incorporated with PLGA microparticles, Biomaterials. 27(28) (2006) 4941-4947

29) E.M. Ooms, E.A. Egglezios, J.G.C. Wolke, J.A. Jansen, Soft tissue response to newly developed calcium phosphate cements, Biomaterials. 24 (2003) 749-757

30) A. Venien, D. Levieux, Differentiation of bovine from porcine gelatins using polyclonal anti-peptide antibodies in indirect and competitive indirect ELISA, J Pharm Biomed Anal. 39 (2005) 418-424

31) H.C. Liang, W.H. Chang, K.J. Lin, H.W. Sung, Genipin-crosslinked gelatin microspheres as a drug carrier for intramuscular administration: in vitro and in vivo studies, J Biomed Mater Res Part A. 65(2) (2003) 271-282

# CHAPTER 9

# CLOSING REMARKS AND FUTURE PERSPECTIVES

## 1. Closing remarks

Calcium phosphate cement is a promising material for bone tissue engineering purposes due to its osteoconductive properties and perfect fit with the surrounding tissue when injected *in situ*. In addition, the calcium phosphate cement as used in this thesis has a very short setting time, good cohesion properties and high compression strength.

### *1.1. Clinical performance*

In view of the above mentioned, the first challenge was to develop a microsphere/calcium phosphate cement composite with a comparable clinical performance as the original calcium phosphate cement. In previous studies, the clinical performance of PLGA microsphere/ calcium phosphate cement composites (PLGA CPCs) with respect to injectability and setting time was not an issue as the applied animal models (cranial defects, rats) acquired preset cement composites. In our studies with PLGA CPC, it was important not to deviate from the optimal L/P ratio (L/P =0.32) of the cement to obtain a microsphere/calcium phosphate cement composite with good setting and cohesion properties as well as high compression strengths. The optimal L/P ratio could be maintained upon incorporation of 10wt% PLGA microspheres and a composite with good injectability properties was achieved. On the other hand, upon the addition of 20wt% of PLGA microspheres L/P ratio had to be increased because the stiff PLGA microspheres affected the injectability properties, which resulted in a significant increase of the final setting time. Further, a 1% solution of $Na_2HPO_4$ was shown to be the best accelerator.

For both gelatin and PTMC microspheres the same mixing parameters were used (low L/P ratio, 1% $Na_2HPO_4$), but still some extra changes were necessary. As gelatin is a hydrogel, microspheres had to be added in a swollen state to the cement to achieve a good distribution of microspheres and to avoid swelling of the microspheres after implantation. Also with respect to drug loading, microspheres had to be swollen first to prevent extensive binding of the protein to the cement. The high compressibility of these swollen microspheres improved injectability of the gelatin microsphere CPCs, but increased the final setting time even more than for the PLGA microspheres. Initial setting time only increased gradually, which enabled us to prepare a >50 vol% gelatin type A microsphere/calcium phosphate cement composite with an initial setting time of only 4 min. Furthermore, setting time of the gelatin microsphere CPCs was highly dependent on the type of microspheres and microsphere size as the use of smaller type B gelatin microspheres resulted in even more problems with cement setting. Incorporation of PTMC microspheres produced similar properties as the gelatin microsphere CPC with respect to setting time. Handling properties with these PTMC CPCs were hampered by the thermoplastic properties of the polymer, which acquired storage and processing of the microspheres in aqueous solution.

Final mechanical properties of the microsphere CPCs (compression strength, E-modulus) were less than the original cement, but still a minimal compressive strength of 14 MPa was obtained. Upon degradation of the microspheres, compression strength decreased in all CPCs. For the PTMC

microsphere CPCs, some improvement of the elastic properties was observed as the strain-at-yield and toughness of the composite increased upon the addition of a higher percentage of PTMC microspheres. After brittle fracture of the cement, microspheres were elongated and kept the individual pieces together.

### *1.2. Degradation*

The *in vitro* and *in vivo* degradation of these microsphere CPCs was also tailored, because microspheres were incorporated with different degradation and erosion mechanisms. Furthermore, the individual polymers as chosen for the preparation of the microspheres (PLGA, gelatin and PTMC) also showed a degradation pattern that can be customized by altering the molecular weight or crosslinking density. Overall, the PLGA microsphere CPC showed the highest applicability as porogen. Degradation rates of the PLGA microspheres were highly dependent on the molecular weight and produced a concomitant porosity through the composite. The production of acidic degradation products appeared even to increase the *in vitro* cement dissolution.

Gelatin microspheres showed a more gradual degradation, i.e. the microspheres degraded from the outside to the center of the composite. Such a degradation mechanism has to be preferred, because it fits to the process of bone formation (ingrowth). On the other hand, a high amount of gelatin microspheres was needed to produce porosity as gelatin degrading enzymes have a strong affinity with the cement. Calcium phosphate precipitate onto the biomimetic gelatin also hampered interconnectivity of the microspheres. The crosslinking density of the gelatin microspheres did not influence *in vitro* degradation inside the composites, but it has to be noticed that non-crosslinked microspheres were not stable upon mixing.

In the *in vitro* study, PTMC CPC showed a similar degradation mechanism as gelatin. The surface erosion of the PTMC microspheres resulted in a fast decrease of the compression strength of the PTMC CPC. However, the *in vivo* degradation was substantially different and was characterized by a gradual decrease in compression strength and slower overall degradation. This discrepancy between *in vitro* and *in vivo* results was due to the high amount of PTMC degrading enzyme as used in the *in vitro* degradation experiment. The molecular weight of the PTMC microspheres did not influence degradation in a high extent, which can be caused by the presence of hydrophilic PVA at the surface of the microspheres.

### *1.3. Drug delivery*

In this study, the different microspheres were also used as a drug delivery vehicle for osteoinductive growth factors. With PLGA, previous studies have already shown to provide a sustained *in vivo* release of growth factor with an additional enhancement of bone ingrowth. The current *in vitro* BSA release study questions whether PLGA microspheres are indeed the most suitable vehicle for drug

delivery, because no retention of BSA structure was observed and release (from the cement) was hampered by the pH decrease.

Gelatin microspheres showed a more favorable *in vitro* release pattern with even a linear sustained release in time, which was tailored by changing the type of gelatin, amount of microspheres and loading mechanism. BSA release from the gelatin type A microsphere CPC, also showed retention of protein structure and substantial higher release efficiencies than the PLGA CPC. Compared to the PLGA CPC, the loading mechanism for the gelatin microsphere CPCs is also more favorable as microspheres are loaded directly before mixing with the cement powder without any substantial loss or exposure to organic solvents. A drawback is the low binding efficiency between the gelatin microspheres and growth factors or drugs, which results in substantial dispersion of the drug through the cement during composite preparation.

PTMC microspheres can also be used as a vehicle for drug delivery, but the present preparation process of the PTMC CPCs where microspheres are stored in aqueous solutions is not optimal for drug entrapment efficiency and has to be altered before further exploration of their use in CPC.

### *1.4. General conclusions*

To conclude, this thesis shows that calcium phosphate cement with various biodegradable microspheres can produce macroporosity during *in vitro/in vivo* degradation and still possesses appropriate clinical handling properties. Furthermore, the *in situ* formation of macroporosity and drug release characteristics of the different microsphere composites can be tailored by choosing a polymer with a different degradation or erosion pattern.

## 2. Future perspectives

In view of the studies performed in this thesis some future perspectives can be given regarding the final application of a microsphere CPC for tissue engineering purposes.

### *2.1. Combinations of different types of microspheres*

The various studies as presented in this thesis have shown that the addition of various types of microspheres into CPC results in a wide range of degradation patterns and alters the handling properties of the CPC. Therefore, by combining different kinds of microspheres into one specific composite it is possible to obtain a composite with customized degradation rates, release characteristics and handling properties. Especially, the following combinations of microspheres are worth further investigation:

- *PLGA and gelatin (type A or B) microsphere CPC*

  A combination of PLGA with gelatin microspheres, as already tested in our *in vivo* experiment can combine the tailored degradation rates of the PLGA with the sustained drug release characteristics of the gelatin. Furthermore, the porosity generated after PLGA degradation can improve gelatin

microsphere degradation and alternatively the remaining gelatin microspheres provide mechanical strength to the inner parts of the cement. Also, such a mix is favorable for the handling properties of the CPC, since the flexible gelatin microspheres will compensate for the decreased injectability of the PLGA microspheres, and even will allow the incorporation of a high amount of large microspheres (several 100 μm's) without the problems of clogging at the syringe opening.

- *PLGA and PTMC microsphere CPC*
  Similar to the previous composite, a combination of PLGA microspheres with PTMC microspheres is an interesting option. The unique elastic properties of the PTMC microspheres will improve the mechanical properties of the CPC.
- *Gelatin type A and gelatin type B microsphere CPC*
  Incorporation of gelatin type A and type B microspheres in one composite can be used for the sustained release of basic and/or acidic growth factors. Also, the opposite charge of the different gelatins can be used to form interconnections between the microspheres.

Another promising combination is the use of different molecular weights of PLGA microspheres. The formation of macroporosity could be modified in this way, but to obtain an interconnective matrix after microsphere degradation the faster degrading microspheres have to be situated at the outside of the composite and the slower degrading microspheres at the center.

### *2.2. Other adjustment of microsphere CPC*

Besides the combination of different types of microspheres, also others adjustments can be made to improve the handling properties, degradation and release characteristics of the microsphere CPCs.

When the material is injected *in situ* the presence of blood and body fluids hampers occasionally the setting reaction and influences the cohesion of the composite. For gelatin microsphere CPC the amount of liquid hardener added during the second step can be decreased within a certain range, which will result in a viscous, but still injectable paste. In case of bleeding at the implant site, cohesion will then be retained as the blood will be adsorbed by the gelatin.

One of the most important parameters for degradation of the included polymeric microspheres and subsequent tissue ingrowth is the interconnectivity of the pores. In addition to the use of a high amount (vol%) of microspheres, interconnections between microspheres (pores) can also be obtained by agglomeration of microspheres. Evidence of this mechanism has been found with (HMW) PTMC microspheres and is probably caused by the plastic deformation of the polymer during cement mixing.

From the gelatin release study, we can also conclude that when the drug is adsorbed onto the cement, the cement scaffold itself can be a suitable drug delivery vehicle. Depending on the final clinical application of the injectable CPC, a possibility is the addition of a growth factor solution after *in situ* initial setting of the material.

### *2.3. Modifications in study design*

Some modifications in the design of the *in vitro* or *in vivo* studies can be made, which will provide more insight in the clinical performance, degradation characteristics and drug release kinetics of these composites.

Previous *in vitro* studies have shown that the used calcium phosphate cement reaches its maximum compression strength after 3 days of incubation in a liquid environment. From the performed *in vivo* study it can be questioned whether this is indeed true in an *in vivo* environment as proteins that are present in the body fluids can interfere with the setting reaction and resulting mechanical properties. A simple test where preset disks or injectable materials are incubated in cell-culture medium or (simulated) body fluid already can provide a lot of information about these issues. Besides compression strength measurements, also other mechanical tests can be performed on the different microsphere CPCs, like three-point bending, which will provide more information about the added (elastic) properties of the microspheres.

Further, a flow perfusion system has to be applied during *in vitro* degradation and release studies to mimic more accurately the *in vivo* conditions. As such a system acquires the use of a high volume of medium, release from these composites can be analyzed by measuring the retention of a (radioactive) labeled drug.

Finally, other *in vivo* investigations are necessary, which include the application of different injectable microsphere CPCs in a wide variety of clinically relevant bone defects. In addition to the histological examination, techniques like gel permeation chromatography (GPC) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) have to be used to evaluate PLGA and PTMC microsphere degradation *in vivo*.

# CHAPTER 10

## SUMMARY AND ADDRESS TO THE AIMS

## 1. Summary and address to the aims

In the field of dentistry, plastic and reconstructive surgery and orthopedics, calcium phosphate ceramics/cements are promising implant materials that are often applied for filling bone defects. Especially calcium phosphate cements have a high potential as next to their osteoconductivity they exhibit a perfect contact with the surrounding tissue when injected *in situ*. A disadvantage is the slow resorbability of these materials, thereby causing difficulties with respect to brittle fracture after longer implantation periods. Tissue engineering is a new field of science that aims to develop implants that can restore, maintain or improve the functions of the original tissue by the use of combining expertise on medical science, biology and engineering. By the use of a construct that is biodegradable, forms a suitable matrix for tissue (in)growth and is a vehicle for growth factors one can synthesize an implant that is gradually replaced by the hosts own tissue thereby prohibiting post-operative failure of the implant. The introduction of macroporosity is one way to make a calcium phosphate cement more suitable for tissue engineering purposes as it promotes bone ingrowth and increases bioresorption of the material. The resulting macroporosity should be interconnected and large enough to guide tissue ingrowth, however, should not decease the clinical performance of the cement with respect to handling properties, setting and mechanical strength.

The main goal of this research was to develop a calcium phosphate cement that is suitable for bone tissue engineering purposes and furthermore still possesses a good clinical performance. This was done by the incorporation of biodegradable microspheres where porosity is formed after microsphere degradation. Secondly, these microspheres can be used as delivery vehicle for osteoinductive drugs.

### *1.1. Injectable PLGA microsphere/calcium phosphate cement composites: physical properties and degradation characteristics*

In chapter 2 we aimed to develop poly(lactic-*co*-glycolic)acid (PLGA) microsphere/calcium phosphate cement composites (PLGA CPCs) with appropriate setting/cohesive properties and good mechanical/physical characteristics. Secondly, we investigated the *in vitro* degradation characteristics of preset samples. Therefore, 10/90 and 20/80 dry wt% PLGA microsphere/calcium phosphate cement composites were prepared as well as CPC controls. Injectability, setting time, cohesive properties and porosity were determined. Also, a 12-week degradation study in PBS (37°C) was performed. Results showed that injectability decreased with an increase in PLGA microsphere content. Initial and final setting time of the PLGA CPC was higher than the original CPC. Porosity of the different formulations was 40.8% (CPC), 60.2% (10/90) and 69.3% (20/80). The degradation study showed distinct mass loss and a pH decrease of the surrounding medium starting from week 6 with the 10/90 and 20/80 formulations, indicating PLGA erosion. Compression strength of the PLGA CPC samples decreased siginificantly in time, the CPC control remained constant. After 12 weeks, both PLGA CPC formulations showed a structure of spherical pores and compression strengths of 12.2MPa (10/90) and 4.3 MPa (20/80) were obtained. Signs of cement degradation were also found with the 20/80

formulation. In conclusion: all physical parameters were well within workable ranges with both 10/90 and 20/80 PLGA microsphere/calcium phosphate cement composites. After 12 weeks the PLGA was totally degraded and a highly porous scaffold remained.

### *1.2. PLGA microsphere/calcium phosphate cement composites for tissue engineering: in vitro release and degradation characteristics*

In chapter 3 we tried to elucidate the drug release mechanism of PLGA CPC. Therefore, both *in vitro* degradation characteristics and drug release pattern of the PLGA CPC were determined using bovine serum albumin (BSA) as a model protein. Two loading mechanisms were applied; BSA was adsorbed onto the microspheres or incorporated into the microspheres during double-emulsion. BSA release from PLGA microspheres and CPC was also measured and used as reference. Results show fast degrading polymer microspheres, which produced a macroporous scaffold within 4 weeks but also showed a concomitant release of acidic degradation products. BSA release from the PLGA CPC was similar to the CPC samples and showed a pattern consisting of a small initial release, followed by a period of almost no sustained release. Separate PLGA microspheres exhibited a high burst release and release efficiency that was higher with the adsorbed samples. For the PLGA CPC samples, the BSA readsorbed to the cement surface after being released from the microspheres. This process was mediated by the pH decrease during microsphere degradation.

### *1.3. Introduction of gelatin microspheres into an injectable calcium phosphate cement*

In chapter 4 we developed gelatin microsphere/calcium phosphate cement composites and investigated their handling properties and *in vitro* degradation characteristics. Therefore, setting time and injectability were determined and an *in vitro* degradation study was performed. Samples were assayed on mass, compression strength, E-modulus and morphology. A supplementary degradation test with gelatin microspheres was performed to investigate the influence of physical conditions inside the cement on microsphere stability. Results showed that the gelatin microsphere CPCs were easy to inject and showed initial setting times of less than 3 min. After 12 weeks *in vitro* degradation no increase in macroporosity was observed, which was supported by the small mass loss and stabilizing mechanical strength. Even a clear densification of the composite was observed. Explanations for the lack of macroporosity were recristallization of the cement onto or inside the gelatin spheres and a delayed degradation of gelatin microspheres inside the scaffold. The supplementary degradation test showed that the pH is a factor in the delayed gelatin microsphere degradation. Also differences in degradation rate between types of gelatin were observed. Overall, the introduction of gelatin microspheres into CPC renders composites with good handling properties, though the degradation characteristics should be further investigated to generate a macroporous scaffold.

### *1.4. Porcine gelatin microsphere/calcium phosphate cement composites: in vitro degradation and protein release*

In chapter 5 a porcine gelatin (type A) microsphere/calcium phosphate cement composite was formulated. Secondly, the *in vitro* degradation and release characteristics of preset composites were investigated. Therefore, the *in vitro* degradation of 10wt% gelatin type A microsphere CPCs (GELA CPCs) was followed for 12 weeks in proteolytic medium. Composites were loaded with bovine serum albumin (BSA) by swelling of gelatin microspheres in a BSA solution for a short (instant) and longer (prolonged) period. *In vitro* BSA-release was analyzed by reversed-phase high performance liquid chromatography (RP-HPLC), structural stability of BSA was monitored by SDS-PAGE. Results of the degradation test for the GELA CPC showed a gradual decrease in mass, compression strength and E-modulus. Morphology investigation showed that degradation of the spheres started at the surface of the composite and gradually proceeded to the inner part. From the release assay it was concluded that the BSA release pattern of GELA CPCs can be altered within certain ranges by changing the loading mechanism. No initial burst was observed and release efficiencies up to 43% after 63 days were obtained. From these results we can conclude that GELA microspheres can be used to generate *in situ* macroporosity into an injectable calcium phosphate cement and tailor release properties from these gelatin microsphere CPC composites.

### *1.5. In vitro growth factor release from injectable calcium phosphate cements containing gelatin microspheres*

In chapter 6 we investigated the *in vitro* growth factor release from various gelatin microsphere CPCs. For this, recombinant human TGF-β1, bFGF and BMP-2 were labeled with $^{125}I$ and loaded onto gelatin type A/type B microspheres for a short (instant) and longer (prolonged) time before mixing them with the cement. Radioactivity of the resulting 5 or 10wt% gelatin microsphere CPC composites was monitored for 6 weeks when subjected to proteolytic medium. Drug-loaded CPC was used as control. Results showed that release pattern/efficiency of gelatin microsphere CPCs and CPC controls was highly dependent on the type of growth factor but unaffected by the amount of growth factor. With gelatin microsphere CPC, release was also dependent on the type of gelatin, total volume of incorporated microspheres and loading method.

### *1.6. Introduction of enzymatically degradable poly(trimethylene carbonate) microspheres into an injectable calcium phosphate cement*

In chapter 7 we developed calcium phosphate cements with incorporated enzymatically degradable PTMC microspheres (PTMC CPCs) and investigated their physical/mechanical properties and *in vitro* degradation characteristics. Therefore, composites were tested on setting time and mechanical strength as well as subjected to phosphate buffered saline (PBS) and enzyme containing medium. PTMC CPCs (12.5 and 25wt%) with molecular weights of 52.7 $kg \cdot mol^{-1}$ and 176.2 $kg \cdot mol^{-1}$ were prepared, which

showed initial setting times similar to the original CPC. Though compression strength decreased upon incorporation of PTMC microspheres, elastic properties were improved as strain-at-yield increased with increasing content of microspheres. Sustained degradation of the microspheres inside the PTMC CPC occurred when incubated in the enzymatic environment, but not in PBS, which resulted in an interconnected macroporosity for the 25 wt% composites.

### *1.7. In vivo degradability of calcium phosphate cement with incorporated biodegradable microspheres*

In chapter 8 we investigated the influence of the microsphere degradation or erosion mechanism on the *in vivo* degradation of microsphere/calcium phosphate cement composites (microsphere CPCs) for tissue engineering. Microspheres composed of poly(lactic-*co*-glycolic acid) (PLGA), gelatin and poly(trimethylene carbonate) (PTMC) were used as models and the resulting microsphere CPCs were implanted subcutaneously for 4, 8 and 12 weeks in the back of New Zealand white rabbits. Besides degradation, the soft tissue response to these formulations was evaluated. After retrieval, specimens were analyzed by physicochemical characterization and histological analysis.

Results showed that all microsphere CPCs exhibited microsphere degradation after 12 weeks of subcutaneous implantation, which was accompanied by a decrease in compression strength. The PLGA microspheres exhibited bulk erosion throughout the whole composite, whereas in the case of gelatin degradation of the microspheres preceded from the outside to the center of the composite. High molecular weight PTMC microspheres exhibited surface erosion, which resulted in decreasing microsphere sizes. All composites showed a similar tissue response with a decreasing capsule thickness over time and a persisting moderate inflammatory response at the implant interface. In conclusion: microsphere CPCs can be used to generate porous scaffolds in an *in vivo* environment after degradation of microspheres with various degradation/erosion mechanisms.

# CHAPTER 11

## SAMENVATTING EN REALISATIE VAN DE DOELSTELLINGEN

## 1. Samenvatting en realisatie van de doelstellingen

Op het gebied van de tandheelkunde, plastische en reconstructieve chirurgie en orthopedie zijn calcium fosfaat cementen/keramieken veelbelovende materialen die vaak worden toegepast voor het opvullen van botdefecten. Met name calcium fosfaat cementen (CPC's) hebben een goede potentie omdat ze naast osteoconductiviteit een perfect contact hebben met het onringende weefsel wanneer ze *in situ* worden geinjecteerd. Een nadeel van deze cementen is de langzame degradatie waardoor problemen kunnen optreden zoals een brosse breuk na langere implantatieperioden. Tissue Engineering is een nieuw wetenschapsgebied waarbij het doel is om implantaten te maken die de functies van het originele weefsel kunnen vervangen, behouden of verbeteren door het samenvoegen van expertise op het gebied van medicijnen en natuurwetenschappen. Door het gebruik van een biologisch degradeerbaar construct dat een geschikte matrix vormt voor weefselingroei en een vector vormt voor groeifactoren, kan men een implantaat maken dat langzaam wordt vervangen door het eigen weefsel en zo post-operatieve complicaties kan voorkomen. Het introduceren van macroporositeit is een manier om calcium fosfaat cement geschikt te maken voor tissue engineering omdat het de ingroei van bot vergemakkelijkt en degradatie versnelt. De verkregen macroporositeit zou hierbij interconnectief en groot genoeg moeten zijn voor botingroei, hoewel, het mag ook niet de performance van het cement verminderen betreffende hanteerbaarheid, uitharding en mechanische sterkte.

Het doel van dit onderzoek was het ontwikkelen van een calcium fosfaat cement dat geschikt is voor tissue engineering en tevens een goede clinische performance bezit. Dit werd bereikt door het incorpereren van biodegradeerbare microsferen in cement, waar porositeit verkregen wordt na degradatie van de microsferen. Tevens kunnen deze microsferen gebruikt worden als vector voor osteoinductieve groeifactoren.

### *1.1. Injecteerbare PLGA microsfeer/calcium fosfaat cement composieten: physische eigenschappen en degradatie karakterististieken*

Het doel van hoofdstuk 2 was het ontwikkelen van een poly(melk-co-glycol)zuur (PLGA) microsfeer/calcium fosfaat cement composiet (PLGA CPC) met geschikte uithardings,- en cohesieve eigenschappen en goede mechanische/ physische karakteristieken. Tevens onderzochten we de *in vitro* degradatie van uitgeharde samples. 10/90 en 20/80 wt% PLGA CPC's en CPC (referentie) werden gesynthetiseerd waarvan de injecteerbaarheid, uithardtijd en cohesieve eigenschappen werden bepaald. Ook werd er een degradatiestudie uitgevoerd in PBS (37°C) voor 12 weken. Resultaten lieten een afname in injecteerbaarheid zien met een grotere hoeveelheid geincorpereerde PLGA microsferen. Initiele en finale uithardtijd van het PLGA CPC waren hoger dan die van het originele CPC. Porositeit van de verschillende

formuleringen was 40.8% (CPC), 60.2% (10/90) en 69.3% (20/80). Na 6 weken liet de degradatiestudie voor beide formuleringen een duidelijke afname in massa zien en pH van het omringende medium, wat indicatief is voor PLGA degradatie. De compressiesterkte van de PLGA CPC samples in de tijd liet een siginificante afname zien, waar de CPC controle constant bleef. Na 12 weken was bij beide PLGA CPC formuleringen een structuur van sferische porien zichtbaar en werden compressiesterktes van 12.2MPa (10/90) en 4.3 MPa (20/80) verkregen. De 20/80 formulering liet ook tekens van cement degradatie zien. In conclusie: alle mechanische/fysische parameters zijn binnen clinisch aanvaardbare waarden voor beide 10/90 en 20/80 PLGA microsfeer/calcium fosfaat cement composieten. Na 12 weken was het PLGA volledig degradadeerd resulterend in een poreuze matrix.

### *1.2. PLGA microsfeer/calcium fosfaat cement composieten voor tissue engineering: in vitro afgifte en degradatie eigenschappen*

In hoofdstuk 3 probeerden we het mechanisme van eiwit/medicijnen afgifte uit het PLGA CPC op te helderen. Daarvoor werden zowel de *in vitro* degradatie eigenschappen bepaald als het eiwit/medicijnen afgifte patroon van het PLGA CPC met behulp van albumine (BSA) als model eiwit. Twee ladingsmechanismen werden toegepast; BSA werd geadsorbeerd op de microsferen of geincludeerd tijdens de microsfeer synthese (dubbele emulsie). BSA afgifte uit PLGA microsferen en CPC werd ook gemeten en gebruikt als referentie. De resultaten lieten microsferen zien die snel degradeerden en een poreuze matrix vormden binnen 4 weken maar tevens zure degradatieproducten produceerden. BSA afgifte uit the PLGA CPC was indentiek aan het CPC en bestond uit een kleine initiele afgifte gevolgd door een periode waarbij de afgifte minimaal was. Afzonderlijke PLGA microsferen lieten een hoge initiele afgifte en efficientie zien, welke het hoogst was bij de samples waar BSA was geadsorbeerd op de microsferen. In het algemeen kan men concluderen dat voor het PLGA CPC, BSA weer adsorbeert op het cementoppervlak na afgifte uit de microsferen. Dit proces wordt gecatalyseerd door de pH afname tijdens de degradatie van de microsferen.

### *1.3. De introductie van gelatine microsferen in een injecteerbaar calcium fosfaat cement*

Hoofdstuk 4 beschijft de ontwikkeling van gelatine microsfeer/calcium fosfaat cement composieten (gelatine microsfeer CPC's), waarbij is gekeken naar de clinische toepasbaarheid en *in vitro* degradatie-eigenschappen van deze materialen. Hiervoor werden de uithardtijd en injecteerbaarheid van het composiet bepaald en werd er een *in vitro* degradatiestudie uitgevoerd waarbij samples werden onderzocht op massa, compressiesterkte, E-modulus en morfologie. Een supplementaire degradatietest met gelatine microsferen werd uitgevoerd om de invloed van fysiche omstandigheden in het cement, zoals pH en calcium concentratie, op de stabiliteit van de microsferen te onderzoeken. Resultaten toonden aan dat het gelatine

microsfeer CPC makkelijk te injecteren was en een initiele uithardtijd had van minder dan 3 minuten. Na 12 weken *in vitro* degradatie was er geen toename in macroporositeit. Dit werd gestaafd door de kleine afname in massa en stabiele mechanische sterkte. Er werd zelfs een duidelijke verdichting van het composiet geconstateerd. De uitleg voor het gebrek aan macroporositeit was kristallisatie van het cement op de gelatine microsferen en een vertraagde degradatie van de microsferen in het cement. De supplementaire degradatietest liet zien dat de pH een belangrijke factor is in deze vertraagde degradatie. Daarnaast werden er ook verschillen in degradatiesnelheid geobserveerd tussen verschillende soorten gelatines. Concluderend, de introductie van gelatine microsferen in CPC levert composieten met een goede hanteerbaarheid, hoewel de degradatie-eigenschappen verbeterd moeten worden om een macroporeuze matrix te produceren.

### *1.4. Gelatine type A microsfeer/calcium fosfaat cement composieten: in vitro degradatie en eiwitafgifte*

In hoofdstuk 5 werd er een gelatine type A microsfeer/calcium fosfaat cement composiet geformuleerd waarvan de *in vitro* degradatie-eigenschappen en het eiwit/medicijn-afgiftepatroon werden onderzocht. Hiervoor werd de *in vitro* degradatie van 10wt% gelatine type A microsfeer CPC (GELA CPC) gevolgd voor in totaal 12 weken in proteolytisch medium. Daarnaast werden composieten geladen met albumine (BSA) door gelatine microsferen te zwellen in een BSA oplossing voor een korte (instantane) en langere (verlengde) periode. *In vitro* BSA-afgifte werd geanalyseerd door reversed-phase high performance liquid chromatography (RP-HPLC), structurele stabiliteit van het BSA werd gevolgd met behulp van SDS-PAGE. Resultaten van de degradatietest voor het GELA CPC vertoonden een geleidelijke afname in massa, compressiesterkte en E-modulus in de loop van de tijd. Morfologisch onderzoek toonde aan dat de degradatie van de microsferen begon aan het oppervlak van het composiet en geleidelijk doorzette naar het midden. Resultaten van het BSA-afgifte experiment lieten zien dat het BSA-afgiftepatroon uit het GELA CPC kan worden gevarieerd door het veranderen van het ladingsmechanisme. Geen initiele afgifte werd geconstateerd en na 63 dagen werden efficienties van 43% verkregen. Gelatine type A microsferen kunnen dus gebruikt worden om *in situ* macroporositeit te verkrijgen in een injecteerbaar calcium fosfaat cement en kunnen tevens gebruikt worden om eiwit/medicijnafgifte te reguleren van deze gelatine microsfeer/CPC composieten.

### *1.5. In vitro afgifte van groeifactoren uit een injecteerbaar gelatine microsfeer/calcium fosfaat cement composiet*

In hoofdstuk 6 werd de *in vitro* afgifte van groeifactoren uit verschillende gelatine microsfeer/calcium fosfaat cement composieten onderzocht. Hiervoor werd recombinant

humaan TGF-β1, bFGF and BMP-2 gelabeled met $^{125}I$ en geladen in gelatine type A/type B microsferen voor een korte (instantane) en langere (verlengde) periode. Radioactiviteit van de resulterende 5 of 10wt% gelatine microsfeer CPC's werd gevolgd voor 6 weken in proteolytisch medium. Groeifactor-geladen CPC werd gebruikt als referentie. Resultaten toonden aan dat het afgifte patroon en de efficientie afhankelijk waren van het type groeifactor maar niet afhankelijk van de hoeveelheid geladen groeifactor. De afgifte uit de gelatine microsfeer CPC's was ook afhankelijk van het type gelatine, het totale volume aan geincorpereerde microsferen en het ladingsmechanisme.

### *1.6. Introductie van enzymatisch degradeerbare poly(trimethyleen carbonaat) microsferen in een injecteerbaar calcium fosfaat cement*

In hoofdstuk 7 werd er een poly(trimethyleen carbonaat) PTMC microsfeer/calcium fosfaat cement composiet geformuleerd waarvan de fysische/mechanische eigenschappen en *in vitro* degradatie eigenschappen werden onderzocht. Hiervoor werden composieten getest op uithardtijd en mechanische sterkte alsook geincubeerd in fosfaat gebufferde zoutoplossing (PBS) en enzym bevattend medium. PTMC CPC's (12.5 en 25 wt%) met molecuulgewichten van 52.7 $kg \cdot mol^{-1}$ en 176.2 $kg \cdot mol^{-1}$ werden gesynthetiseerd met een uithardtijd identiek aan het originele CPC. Alhoewel de compressiesterkte afnam met de introductie van PTMC microsferen, vertoonden de elastische eigenschappen een verbetering doordat de strain-at-yield toenam met een toename in hoeveelheid geincorpereerde microsferen. Geleidelijke degradatie van de microsferen in het PTMC CPC vond plaats in het enzymatisch milieu, maar niet in PBS, resulterend in een interconnectieve, macroporeuze matrix voor de 25wt% composieten.

### *1.7. In vivo degradatie van calcium fosfaat cement geincorpereerd met biodegradeerbare microsferen*

In hoofdstuk 8 is gekeken naar de invloed van het microsfeer degradatie/erosie mechanisme op de *in vivo* degradatie van microsfeer/calcium fosfaat cement composieten voor tissue engineering. Microsferen gemaakt van PLGA, gelatine en PTMC werden hiervoor gebruikt als model en subcutaan geimplanteerd voor 4, 8 en 12 weken in een konijnenstudie. Behalve degradatie werd ook de (zachte) weefsel reactie op deze formuleringen onderzocht. Na het verkrijgen van de specimens werden ze geanalyseerd op physicochemische eigenschappen en histologie.

Resultaten lieten zien dat alle formuleringen degradatie vertoonden na 12 weken subcutane implantatie. Het PLGA CPC vertoonde bulkerosie van de microsferen door het hele implantaat, terwijl gelatine microspheren eerst degradeerden aan de oppervalkte van het

composiet. Hoog moleculair gewicht PTMC microsferen vertoonden oppervlakte-erosie, resulterend in een afname van de microsfeerdiameter. Uit histologisch onderzoek kwam naar voren dat alle composieten een vergelijkbare weefselreactie vertoonden met een afname in kapseldikte en een gematigde inflammatiereactie op het implantaatoppervlak. Concluderend, de onderzochte microsfeer/CPC formuleringen kunnen gerbruikt worden om een poreuze matrix te genereren na microsfeer degradatie in een *in vivo* omgeving.

# Dankwoord

Na vier jaar promotieonderzoek en een stage is het nu eindelijk tijd om weer een nieuwe stap te zetten in mijn loopbaan. Het werk dat ik de afgelopen jaren heb verricht in Nijmegen kon ik nooit doen zonder de intellectuele en morele hulp van vele mensen welke ik allemaal zou willen bedanken. Ook op persoonlijk vlak heb ik me de afgelopen jaren kunnen ontwikkelen en heb ik me altijd op mijn gemak gevoeld getuige het luidkeels zingen op het lab en vlinderfoto's welke de koffieruimte hebben gesierd.

Prof J.A. Jansen, beste John, een goedlopend onderzoek heeft een persoon nodig die een visie heeft over het geheel en alles de goede richting in baant. De afgelopen vier jaar heb ik veel vrijheid gehad waardoor ik heb geleerd zelf mijn onderzoek te managen. Ook ben ik zeer tevreden over alle presentaties die ik heb mogen houden. Bedankt voor het vertrouwen dat je me hebt geschonken.

Dr. J.G.C. Wolke, Joop, zonder te overdrijven durf ik te zeggen dat je een belangrijke spil vormt voor al het onderzoek bij Biomaterialen. Hoe dat je alles voor elkaar krijgt dat verbaast me nog steeds, zeker nu ik zelf ben begonnen met het begeleiden van studenten. Ik heb veel gehad aan onze dagelijkse gesprekken en discussies of ze nu over onderzoek gingen, gepubliceerde artikelen of onze gezamelijke hobby. Dank voor alles!

Dr. A.G. Mikos, dear Tony, thanks for the opportunity you gave me upon visiting your lab. Next to the world of information I received during my stay, I made good friends with the students and staff at the lab. I also greatly appreciate all the instructions you gave me upon writing my papers.

Dr. Z. Zhang, Zheng, when we met in Lunteren I was sure that our co-operation would lead to some great papers, and I was right. I greatly appreciate all the work you did and remember our montly meetings at the Deventer-station. I furthermore also would like to acknowledge the input of your supervisors, Dirk Grijpma and Jan Feijen.

Dr. O.C. Boerman, beste Otto, ik ben zeer tevreden over het onderzoek dat ik in het lab van nucleaire geneeskunde heb mogen uitvoeren en de hulp die ik daarbij van jou, Huub, Cathelijne en Ingrid heb ontvangen.

Natuurlijk wil ik mijn collega's in het "New Bone" project Dennis en Esther ook in het zonnetje zetten. Ik heb veel profijt gehad van jullie biologische en medische achtergrond, wat mij nieuwe ideëen gaf over het ontwerp van een ideaal botimplantaat, welke ik dan ook veelvuldig met jullie uitwisselde. Daarnaast was het ook gewoon fijn om even bij te kletsen, de dropjespot te legen of wanneer ik mijn administratie weer eens niet op orde had. Ook wil ik met name Lise, Hong-Bing, Juliette en Frank bedanken voor de belangrijke bijdrage die ze hebben geleverd aan mijn onderzoek.

Ik heb veel te danken aan alle analisten in het lab. Anja, ook al heb ik wel eens bonje met je gehad bij het schoonmaken van het lab, ik kon altijd wel terecht met mijn vragen, bestellingen en ook voor advies als ik met iets zat. Ook tijdens de vreemde gebeurtenissen omtrent de pH-meter en vriesdroger heeft het lab altijd als een tierelier gelopen wat voor een groot deel op jouw conto komt. Ook wil ik Vincent, Natasja, Martijn, Jurgen en Jacky bedanken voor al hun expertise en hulp tijdens de verschillende experimenten.

De afgelopen vier jaar heb ik vele kamergenoten gehad, welke elkaar soms in snel tempo afwisselden. Ik veel gehad aan onze dagelijkse gesprekken wanneer ik het lezen weer eens beu was of wanneer ik een engelse vertaling voor een woord/zin zocht.

Wat ik vooral zal missen, dat zijn de dagelijkse beschouwingen over het nieuws (krantjes!), gebeurtenissen of sportevenementen tijdens de ochtend, lunch en middagpauze. Ook heb ik veel plezier beleefd aan onze jaarlijkse uitjes, Lunteren, bowlingavonden en borrelavonden bij St. Anneke en congresbezoeken. Adelina, Adnan, Afsheen, Amit, Anja, Anne, Asuka, Bas, Bamboe Li, Betsy, Corinne, Daniel, Dennis, Dimitris, Edwin (2x), Ellart, Ellen, Elisabeth, Esther, Fang, Frank, Gert, Hans, Henriette, Herma, Hong-Bing, Ika, Jacky, Jeroen, Jochem, John, Joop, Juliet, Juliette, Jurgen, Ken, Kim, Lise, Manal, Marijke, Marjon, Martijn, Meike, Mirjam, Natacha, Nelleke, Olga, Peter, Pia, Quinten, Remco, René, Rudiger, Ruud, Sander (2x), Theresa, Vincent, Walter, Wang, Waquas, Weibo, Xuechao, Yonggang, Zarana, Zoe, dank daarvoor!

Naast mijn werk, wil ik mijn beste vrienden (en paranimfen) Sebastiaan en Ruud van harte bedanken voor alle kameraadschap die ik heb mogen ontvangen in de afgelopen jaren en voor de fantantische zomervakanties en wintersport!

Tenslotte wil ik mijn familie en vooral mijn ouders bedanken voor alles, wanneer ik weer eens laat thuis was van het werk of als de bus niet op tijd was stonden ze altijd voor me klaar!

# Curriculum Vitae

Woutherus Johannes Elisabeth Maria (Wouter) Habraken was born on the 4th of December 1979 in Tilburg, the Netherlands. After finishing secondary school at the Durendael Lyceum in Oisterwijk, he started his study of Chemical Engineering at the Eindhoven University of Technology in September 1998 where he received a scholarship of the Dutch Society of Chemical Industry (VNCI) in 2000. In 2002 he performed a research internship at the group of Biomaterials and Periodontology under supervision of dr. X.F. Walboomers at the Radboud University Nijmegen Medical Center, with the subject "Stretch-mediated responses of osteoblast-like cells cultured on titanium coated implants in vitro". After finishing his Master thesis at the Department of Environmental Technology under supervision of prof. F.J.J.G. Janssen in 2003, he returned to the group of Biomaterials and Periodontology under supervision of prof. J.A. Jansen where from November 2003-2007 he did his PhD research on the development of injectable calcium phosphate cement for bone tissue engineering applications. During his PhD-research he visited the group of Bioengineering at Rice University, Houston (dr. A.G. Mikos) in 2004 where he gained experience about biodegradable microcarriers for drug release. At present, he is working as a post-doc at the department of Solid Materials and Interface Science at the CryoTEM research facility of the Eindhoven University of Technology where he is studying the biomineralization of calcium phosphate.

# List of publications

## Publications related to this thesis

1. W.J.E.M. Habraken, J.G.C. Wolke, J.A. Jansen, Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering, Adv Drug Deliv Rev. 59(4-5) (2007) 234-245

2. W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics, J Biomater Sci Polym Ed. 17 (2006) 1057-1074

3. W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, PLGA microsphere/calcium phosphate cement composites for tissue engineering: *in vitro* release and degradation characteristics, J Biomater Sci Polym Ed. Accepted for publication December 11, 2007

4. W.J.E.M. Habraken, L.T. de Jonge, J.G.C. Wolke, L. Yubao, J.A. Jansen, Introduction of gelatin microspheres into an injectable calcium phosphate cement, J Biomed Mater Res Part A., published online January 14, 2008

5. W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Porcine gelatin microsphere/calcium phosphate cement composites: *in vitro* degradation and protein release, J Biomed Mater Res Part B: Appl Biomater., submitted (2007)

6. W.J.E.M. Habraken, O.C. Boerman, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, *In vitro* growth factor release from injectable calcium phosphate cement containing gelatin microspheres, J Biomed Mater Res Part A., submitted (2007)

7. W.J.E.M. Habraken, Z. Zhang, J.G.C. Wolke, D.W. Grijpma, A.G. Mikos, J. Feijen, J.A. Jansen, Introduction of enzymatically degradable poly(trimethylene carbonate) microspheres into an injectable calcium phosphate cement, Biomaterials. 29(16) (2008) 2464-2476

8. W.J.E.M. Habraken, H.B. Liao, Z. Zheng, J.G.C. Wolke, D.W. Grijpma, A.G. Mikos, J. Feijen, J.A. Jansen, *In vivo* degradability of calcium phosphate cement with incorporated biodegradable microspheres, Adv Mater., submitted (2008)

9. W.J.E.M. Habraken, O.C. Boerman, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Release of growth factors from gelatin microsphere/CaP composites, Key Eng Mat. 361-363I (2007) 527-530

10. W.J.E.M. Habraken, J.G.C. Wolke, A.G. Mikos, J.A. Jansen, Injectable PLGA microsphere/calcium phosphate cements: physical properties and degradation characteristics, Eur Cell Mater. 11 (Suppl 1) (2006) 7

11. D.P. Link, J. van den Dolder, J.J.J.P. van den Beucken, W.J.E.M. Habraken, A. Soede, O.C. Boerman, A.G. Mikos, J.A. Jansen, Evaluation of an orthopically implanted calcium phosphate cement containing gelatin microparticles, J Biomed Mater Res. In press (2008)

## Other Publications

12. X.F Walboomers, W.J. Habraken, B Feddes, L.C. Winter, J.D. Bumgartner, J.A. Jansen Stretch-mediated responses of osteoblast-like cells cultured on titanium-coated substrates in vitro. J Biomed Mater Res Part A 51 (2004) 131-139

# Color Figures

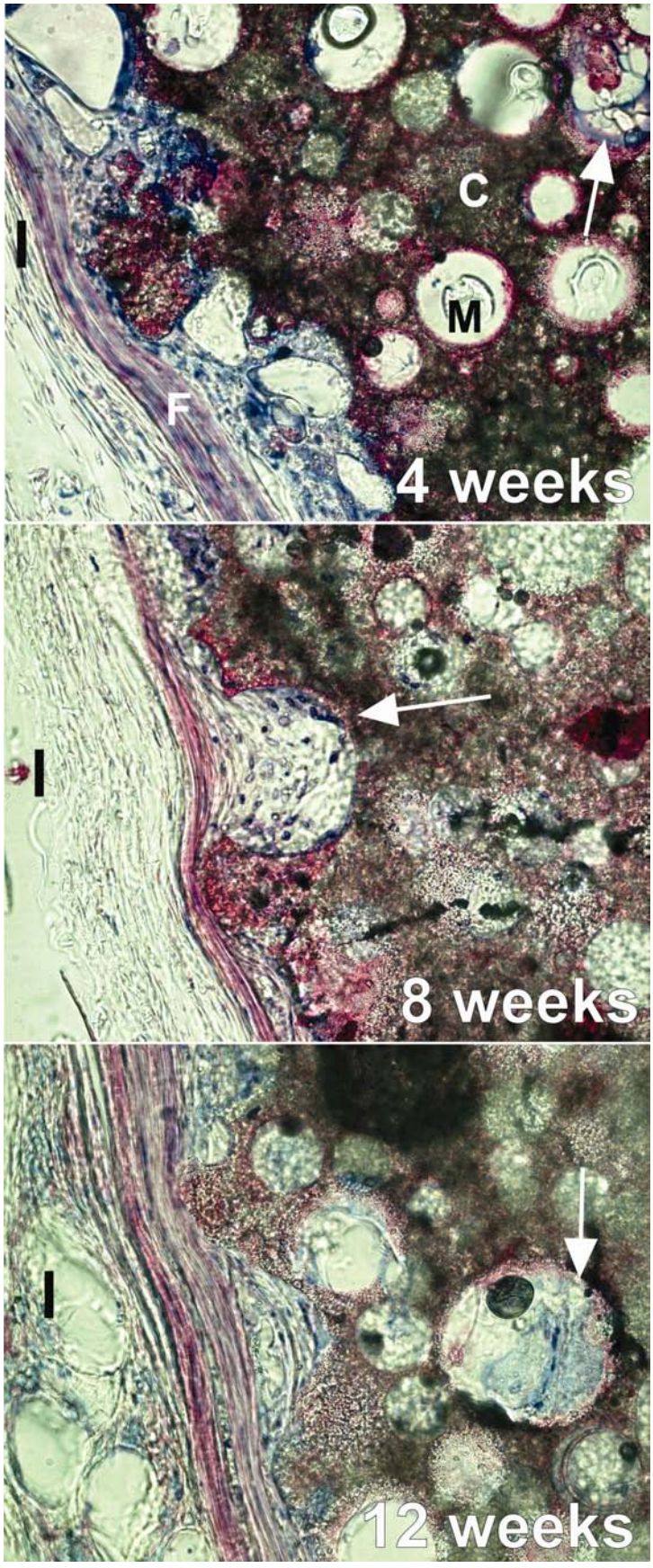



**Figure 9A.** Histological sections PLGA CPCs at 4, 8 and 12 weeks of subcutaneous implantation, C = cement, M = microsphere, F = fibrous capsule, arrows denote tissue ingrowth in microspheres (bar represents 25μm)

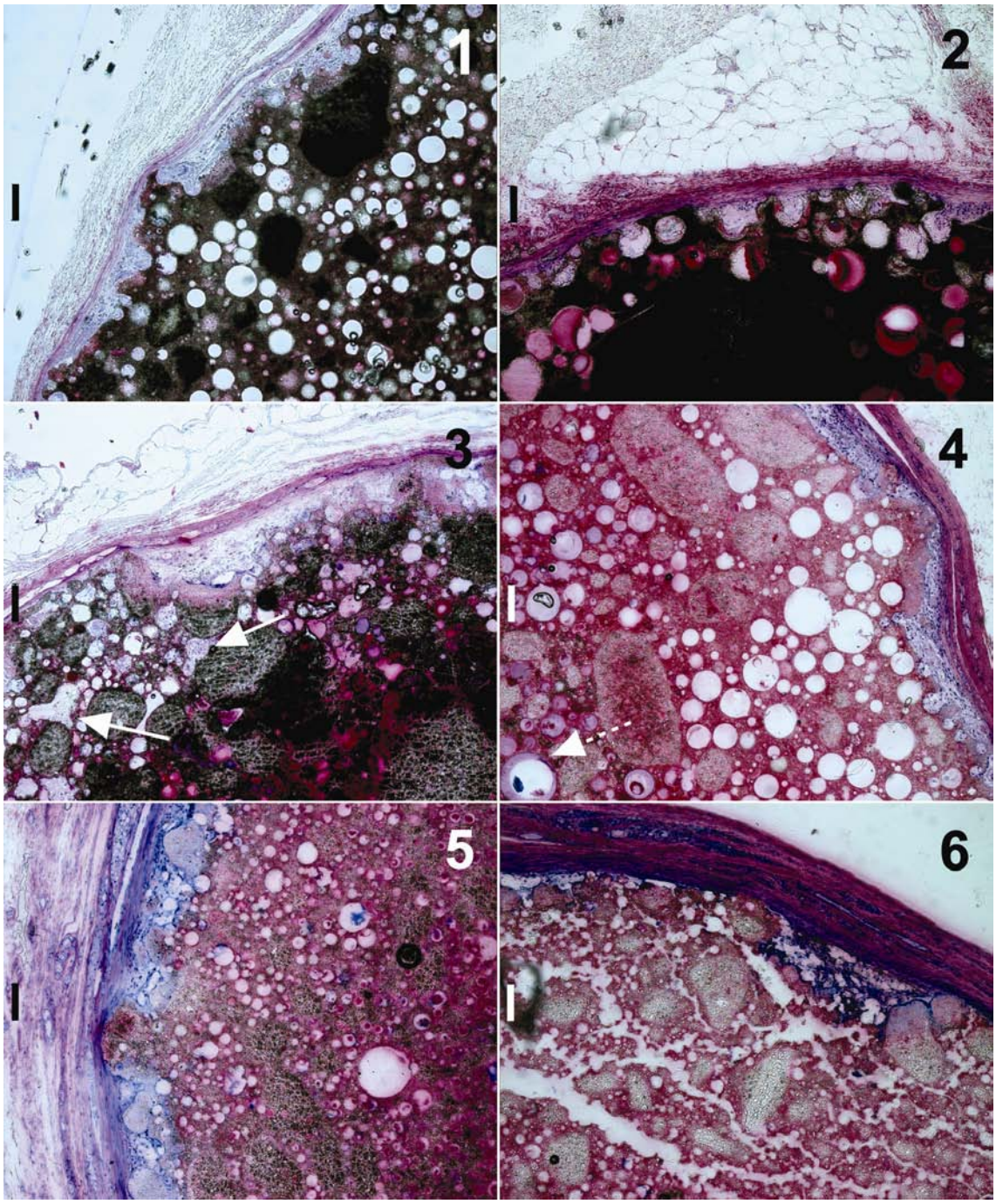



**Figure 9B.** Histological sections of microsphere CPCs after 12 weeks of implantation; PLGA CPC (1), GELA CPC (2), GELB CPC (3), PLGA/GELA CPC (4), LMW PTMC CPC (5) and HMW PTMC CPC (6), arrows denote tissue ingrowth with GELB CPC, dashed arrow denotes shell gelatin microsphere (bar represents 100 μm).