



**POLIANA
POLLIZELLO LOPES**

**CIMENTOS ÓSSEOS ACRÍLICOS MODIFICADOS
COM ENCHIMENTO BIOATIVO E BIODEGRADÁVEL**

**ACRYLIC BONE CEMENTS MODIFIED WITH
BIOACTIVE AND BIODEGRADABLE FILLERS**



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Biomédica, realizada sob a orientação científica da Doutora Maria Helena Figueira Vaz Fernandes, Professora Associada do Departamento de Engenharia Cerâmica e do Vidro da Universidade de Aveiro

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palavras-chave

Cimento ósseo acrílico, vidro bioativo, compósitos, polímero biodegradável, liberação de fármaco, ibuprofeno, resposta celular.

resumo

O cimento ósseo acrílico é o único material utilizado para a fixação de próteses em cirurgias ortopédicas, surgindo como uma alternativa às técnicas não cimentadas. Cerca de um milhão de pacientes são anualmente tratados para a substituição total da articulação do quadril e do joelho. Com a maior expectativa de vida da população e o aumento do número de cirurgias realizadas por ano espera-se que o uso do cimento ósseo aumente substancialmente.

A fraca ligação do cimento ao osso é um problema comum que pode causar perda asséptica da prótese. Assim, torna-se necessário investir no desenvolvimento de cimentos ósseos alternativos que permitam promover maior estabilidade e melhor desempenho do implante.

O principal objetivo desta tese foi desenvolver um cimento ósseo bioativo, capaz de ligar-se ao osso, com propriedades melhoradas relativamente aos sistemas convencionais. A preparação dos materiais foi realizada por dois processos diferentes, a polimerização por via térmica e a polimerização por via química.

Inicialmente, utilizando o processo térmico, foram desenvolvidos compósitos de PMMA-co-EHA reforçados com vidro de sílica (CSi) e vidro de boro (CB) e comparados em termos do seu comportamento *in vitro* em meio acelular e celular. A formação de precipitados de fosfato de cálcio foi observada sobre a superfície de todos os compósitos indicando que estes materiais são potencialmente bioativos. Em relação à avaliação biológica o CSi demonstrou um efeito indutor da proliferação das células. As células apresentaram uma morfologia normal e alta taxa de crescimento quando comparadas com o padrão de cultura. Por outro lado ocorreu inibição da proliferação celular para o CB provavelmente devido à sua elevada taxa de degradação, levando a uma elevada concentração de íons de B e de Mg no meio de cultura.

O efeito do vidro nos cimentos curados por via química, incorporando um activador de baixa toxicidade, também foi avaliado. Os resultados sugerem que as novas formulações podem diminuir o efeito exotérmico na cura do cimento e melhorar as propriedades mecânicas (flexão e compressão). Outro estudo conduzido neste trabalho explorou a possibilidade de incorporar ibuprofeno (fármaco anti-inflamatório) no cimento, dando origem a um material capaz de ser simultaneamente, bioativo e promotor da liberação controlada de fármacos. Neste contexto foi evidenciado que o desempenho do cimento desenvolvido pode contribuir para minimizar o processo inflamatório associado a uma cirurgia ortopédica.

Finalmente, a fase sólida do cimento ósseo bioativo foi modificada por diferentes polímeros biodegradáveis. A adição deste enchimento deu origem a um cimento parcialmente biodegradável que pode permitir a formação de poros e o crescimento ósseo para o interior do cimento, resultando numa melhor fixação da prótese.

keywords

Acrylic bone cement, bioactive glass, composites, biodegradable polymer, drug delivery, ibuprofen, cell response.

abstract

Acrylic bone cement is the only material currently used for anchoring the prosthesis in orthopaedic surgery, being an alternative to non-cemented techniques. About one million patients worldwide are treated annually for total replacement of hips and knee joints. With the longer life expectancy of the population, and the increasing number of surgeries performed every year, the use of acrylic bone cements is expected to rise substantially.

The non bone bonding capability of the cement is a common problem which can cause aseptic loosening of the prosthesis. Thus, alternative cements must be developed to provide higher stability and better performance of the implant.

The main objective of this thesis was to develop a bioactive bone cement, with bone bonding capability, and better properties than the conventional cement. Two different methods of preparation were used in this study, polymerization by chemical route (self-cured) and polymerization by thermal route (heat cured). Initially, through the thermal route, PMMA-co-EHA composites filled with a silicate glass (CSi) and a borate glass (CB) were developed and compared in terms of their in vitro behaviour, both in acellular and in cellular media. The growth of spherical calcium phosphate aggregates was observed in acellular medium on all composite surfaces indicating that these materials became potentially bioactive. Considering the biological assessment, the CSi demonstrated an inductive effect on the proliferation of cells. The cells showed a normal morphology and high growth rate when compared to standard culture plates. On the other hand, inhibition of cell proliferation occurred in the CB probably due to its high degradation rate, leading to high B and Mg ionic concentration in the cell culture medium.

The effect of glass in self cured cements, incorporating an activator of reduced toxicity, was also assessed in this work. The results suggested that the new formulations may lessen the exothermal effect on curing and improve the mechanical properties (bending and compressive). Another study conducted in this thesis explores the possibility of incorporating ibuprofen (anti-inflammatory drug) into the cement, aiming the development of a composite that simultaneously show bioactive behaviour and controlled drug release . It was evidenced that, regarding the drug release, the performance of the developed cements can contribute to blunt the inflammatory process associated to an orthopedic surgery.

Finally the solid phase of the bioactive self-curing acrylic cements was modified by different biodegradable polymers. The addition of the biodegradable fillers made the cement partially degradable, which could allow the formation of pores and the ingrowth of bone to the interior of the cement, resulting in a stronger fixation of the prosthesis.

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LIST OF ABBREVIATIONS

AA	Acrylic acid
ALP	Alkaline phosphatase
ANP	Acryloyl-N-phenylpiperazine
ASTM	American society for testing and materials
AW-GC	Apatite and wollastonite glass-ceramic
BaSO ₄	Barium sulphate
BBC	Bioactive bone cement
BET	Brunauer–Emmett–Teller method
BIEM	2-(2-bromoisobutyryloxy) ethyl methacrylate
Bis-GMA	Bisphenol- α -glycidyl methacrylate
BPEM	2-(2-bromopropionyloxy) ethyl methacrylate
BPO	Benzoyl peroxide
BZN	4,4-bis-dimethylamino benzydrol
CLSM	Confocal laser scanning microscopy
CS NP	Chitosan nanoparticles
DML	4-N,N-Dimethylaminobenzyl laurate
DMOH	N,N-dimethylaminobenzyl-alcohol
DMT	N,N-dimethyl-4-toluidine
DSC	Differential scanning calorimetry
EDS	X-ray spectroscopy
EHA	2-ethyl hexylacrylate
HA	Hydroxyapatite
HCA	Hydroxycarbonate apatite
HDBC _s	Hydrophilic, partially degradable and bioactive cements
HEMA	Hydroxyethyl methacrylate
¹ H NMR	Proton nuclear magnetic resonance
HOB	Human osteoblast-like cells
HQ	Hydroquinone
IB	Ibuprofen
ICP	Inductively coupled plasma
IHQM	2,5-diiodo-8-quinolyl methacrylate
IPMA	4-iodophenol methacrylate
ISO	International standard organization

LD ₅₀	Lethal dose, 50%
α -MEM	α -Minimal Essential Medium
4-META	4-methacryloxyethyl trimellitate anhydride
MMA	Methyl methacrylate
MNP	Methacryloyl-N-phenylpiperazine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCL	poly(ϵ -caprolactone)
PEMA _n BMA	poly(ethylmethacrylate- <i>n</i> -butylmethacrylate)
PHAs	poly(hydroxyalkanoates)
PHB	poly(3-hydroxybutyrate)
PHBV	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PLA	poly-L-lactic acid
PMMA	poly(methyl methacrylate)
PPF	poly(propylene fumarate)
PTFE	poly(tetrafluoroethylene)
QCS NP	Quaternary ammonium chitosan derivative nanoparticles
SBF	Simulated body fluid
SCA	Corn starch/cellulose acetate blends
SEM	Scanning electron microscopy
SSA	Specific surface area
TCP	Tricalcium phosphate
α -TCP	α -tricalcium phosphate
β -TCP	β -tricalcium phosphate
T _g	Glass transition temperature
THA	Total hip arthroplasty
THR _s	Total hip replacements
TKR _s	Total knee replacements
T _m	Melting temperatures
T _{max}	Maximum temperature
TMS	Tetramethylsilane
TPB	Triphenyl bismuth
TPS _s	Thermoplastic starches
t _{set}	Setting time

WL	Weight loss
WU	Water uptake
Xc	Degree of crystallinity
XRD	X-ray diffraction
ZrO ₂	Zirconium dioxide

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CHAPTER 1

HISTORY AND CURRENT STATE OF ACRYLIC BONE CEMENTS

HISTORY

Bone cements are substances used to fix prosthesis to the bones often in joint replacements surgeries and to repair damaged or diseased areas of bones. Most of the bone cements commercially available and currently used in orthopedic procedures are acrylic cements. The basic component of the acrylic bone cement is methyl methacrylate (MMA) which is an ester and can polymerize to form poly(methyl methacrylate) (PMMA). Large scale chemical synthesis of MMA was achieved in the 1920s in the laboratories of Rohm and Haas, and one of the first biomedical applications of PMMA was the fabrication of dentures in 1935 [1, 2]. In the same period (1936) the company Kulzer found that prepolymerized PMMA powder (poly(methyl methacrylate)) could be partly dissolved in MMA, forming a dough that hardens when benzoyl peroxide (BPO) is added and the mixture is heated to 100 °C in a stone mould. Kulzer is at present the producer of the Palacos®, a commercial acrylic bone cement used in orthopaedic surgeries.

The first clinical use of this dough was in an attempt to close cranial defects in monkeys in 1938. Seven years later it was discovered that the polymerization of MMA could occur by itself at room temperature if a tertiary amine (N,N-dimethyl-4-toluidine, DMT) was added, leading to the establishment of a protocol for the chemical production of acrylic bone cements in 1943 [3, 4].

PMMA was first introduced in the orthopedic surgery by Dr. Jean Judet and his brother, Dr. Robert Jude. The Judet brothers developed a hip prosthesis made from PMMA, which was implanted in 1946 [5]. In 1951 Kaier and Jansen in Copenhagen were the first to use PMMA bone cements for the fixation of acrylic cups to the subchondral bone of the femoral head [6]. Nevertheless, it was Sir John Charnley who popularised their use in 1958 and presented the preliminary results of a new method for the fixation of joint prostheses to bone. The idea was to distribute the contact stresses between the implant and the bone over a large area by means of acrylic bone cement. This idea represented an important breakthrough in the field of orthopaedics and led to the development of a worldwide successful technique. The main advantages of the cemented prostheses lay in the excellent primary fixation, in the even load distribution between the implant and the bone, and in the fact that the technique allows a fast recovery of the patient [7].

The addition of antimicrobial agents to acrylic bone cements began as early as 1970. Engelbrecht and Buchholz started investigations on PMMA cement to determine its suitability as a drug delivery system [8-10]. Since 1972, radiopaque materials, like barium sulphate (BaSO₄) or

zirconium dioxide (ZrO_2), have been added to the bone cement in order to provide radio-opacity [11]. In the 1980s acrylic bone cements were also introduced to treat vertebral compression fractures caused by osteoporosis, skeletal metastases and angiomas [12].

Since then many types of bone cements have been developed. Nowadays, there are over 30 commercially available acrylic bone cement brands approved by the relevant regulatory authorities (such as the Food and Drug Administration, FDA, in the US and the Medical Devices Agency in the UK), for use in cemented arthroplasties [13]. In Portugal, the INFARMED is the Portuguese Regulatory Agency for pharmaceuticals, which is the institution in charge of guaranteeing that the legal requirements for the marketing of medicines are met.

BIOMEDICAL APPLICATIONS

The PMMA gained its popularity during World War II as a polymer for biomedical applications, when polymer fragments accidentally implanted in the eyes and other body tissues of pilots during aircraft crashes did not cause damage to the body [14]. When used for orthopedic applications, certain additions are made to PMMA and thus it receives the name of bone cement.

Bone cements have been used as a fixation medium in a number of joint replacements including knee, hip, shoulder, elbow, ankle, and wrist replacements. In recent years, its application has been expanded to vertebroplasty, a procedure in which cement is injected percutaneously into the vertebral body in order to stabilize fractures that occur primarily as a result of osteoporosis. Another variation of this procedure is khyphoplasty, during which a balloon is inserted percutaneously into the vertebral body, inflated to restore the height of the compressed vertebrae and subsequently filled with injected bone cement to stabilize the fracture. These newer applications of bone cement have been successful in relieving pain and restoring vertebral strength and function [15, 16]. Some applications of bone cements are depicted in Figure 1.

In 2002, Khan et al. [17] conducted a systematic review of the literature on treating patients with displaced intracapsular femoral neck fractures. The authors concluded that the publications tended to support the use of cemented hemiarthroplasty, suggesting a lower revision rate, less thigh pain and better mobility in patients whose prosthesis was cemented.

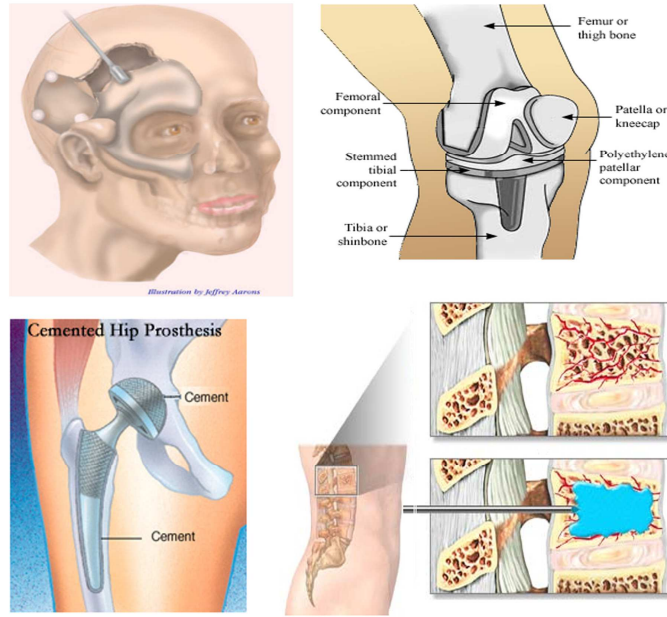


Figure 1: Biomedical applications of a bone cement.

Nowadays the major orthopaedic surgical procedures are total hip and knee joint replacements with 1 million performed worldwide annually. A large proportion of these are anchored to the contiguous cancellous bone in an acrylic bone cement bed [13]. The clinical success rate for cemented implant with 15 years exceeds 90%, especially those of the hip and knee in patients aged over 50 years [18]. In developed countries the acrylic bone cements are used in more than 90% of total hip surgeries [19, 20]. In Sweden, over the period 1979–2000, about 97% of primary total hip replacements (THRs) were cemented [21], and in the United States, 77% of primary total knee replacements (TKRs) were cemented. The majority of total replacements of other joints is also cemented. With the “graying” of the populations in many countries it is expected that the use of acrylic bone cements rise substantially [13].

Total Hip Replacement

A large range of rotary motion is permitted at the hip due to the fitting between femur and pelvis; the top of the femur terminates in a ball-shaped head that fits into a cup-like cavity (the acetabulum) within the pelvis. This joint is susceptible to fracture, which normally occurs at the narrow region just below the head, through the femoral neck. The hip may also become diseased by osteoarthritis; in this case small lumps of bone form on the rubbing surfaces of the joint, which causes pain as the head rotates in the acetabulum and a joint replacement is necessary. Damaged and diseased hip joints have been replaced with artificial joint successfully [22].

One of the major issues confronting contemporary hip surgeons is the choice of fixation method. There is no consensus among orthopedists regarding the appropriate conditions for

prosthesis fixation. However, neither cemented nor uncemented fixation excludes the likelihood of prosthesis loosening [23]. A schematic diagram of the total hip replacement is presented in Figure 2.

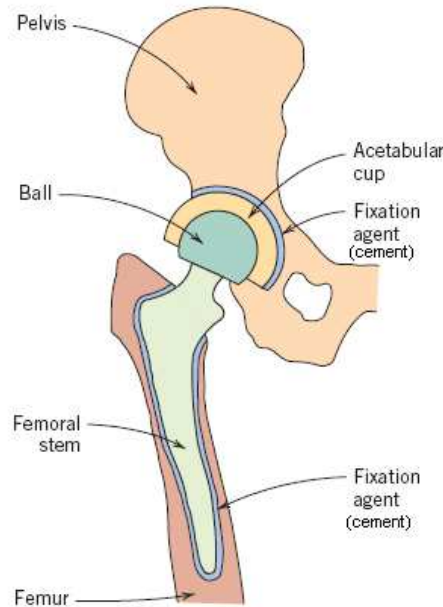


Figure 2: Components of the total hip arthroplasty (THA) system [22]

The hip implant fixation can be cemented, uncemented or hybrid [24, 25].

Cemented fixation: The acrylic bone cement is used to hold the femoral and acetabular components in place. The cemented hip replacement relies on a stable interface between the prosthesis-cement-bone resulting in a faster rehabilitation. Although cemented implants have a long and distinguished track record of success, they are not ideal for everyone. This fixation method is more commonly recommended for older patients, for patients with conditions such as rheumatoid arthritis, and for younger patients with compromised health or poor bone quality and density.

Uncemented fixation: The fixation is made through direct contact to bone without the use of cement. The implants are textured or have a surface coating (osteoconductive coating) providing bone growth into their surface. In general, these designs are larger and longer than those used with cement. Because they depend on new bone growth for stability, uncemented implants require a longer healing time than cemented replacements. This method is most often recommended for younger, more active patients and patients with good bone quality where bone ingrowth into the components can be predictably achieved.

Hybrid fixation: A hybrid total hip replacement has one component, usually the acetabular socket, inserted without cement, and the other component, usually the femoral stem, inserted with cement. This technique was introduced in the early 1980s.

The superiority of either fixation method has not been proved conclusively because of the influences of confounding variables, such as patient age, sex, body weight, and diagnosis [26].

Most of the literature showed that better short and mid-term clinical and functional outcomes could be obtained from cemented femoral fixation than from uncemented femoral fixation [23]. Recent meta-analyses also support superior results of cement fixation when compared to uncemented fixation in large subsets of patient populations [27]. Table 1 presents the rate of revision prostheses according to the fixation method, which also proves that the cemented fixation still shows statistically the best results in terms of the whole THA population. In accordance still with the table uncemented prostheses have the worst performing, resulting in a higher incidence of revision for the studied period [28, 29].

Table 1: Revision rates for the fixation methods used in THA [28].

Revision rates by prosthesis type at one, three and five years for primary hip replacement procedures, undertaken between 1st April 2003 and 31st December 2009.				
Prosthesis type	Number of patients	1 year	3 years	5 years
Cemented	99,359	0.6%	1.4%	2.0%
Uncemented	62,937	1.3%	2.5%	3.4%
Hybrid	31,662	0.9%	1.8%	2.7%

TYPICAL COMPOSITIONS

A typical acrylic bone cement is self-polymerising and consists of two components, a liquid monomer (methyl methacrylate, MMA) and a powder component (polymethylmethacrylate, PMMA). The two components are mixed in the appropriate proportions to form the bone cement. Other additives are included in these components for specific purpose [7, 30].

The monomer contains:

- Hydroquinone (HQ), an inhibitor, which prevents the monomer from prepolymerising spontaneously,
- N-N dimethyl-4-toluidine (DMT), an activator/accelerator, which speeds up the polymerisation reaction.

The powder component contains:

- Benzoyl peroxide (BPO) that acts as initiator, producing free radicals when it reacts with the DMT promoting the polymerization of MMA at room temperature.

- Radiopaque agent, either barium sulphate (BaSO_4) or zirconium dioxide (ZrO_2), which allows the bone cement to be observed on x-rays.

A number of commercial formulations can also include an antibiotic, such as gentamicin sulphate, that provides prophylaxis against infections, which can occur during surgery [8, 31]. Figure 3 illustrates the chemical structure of the main components of an acrylic cement.

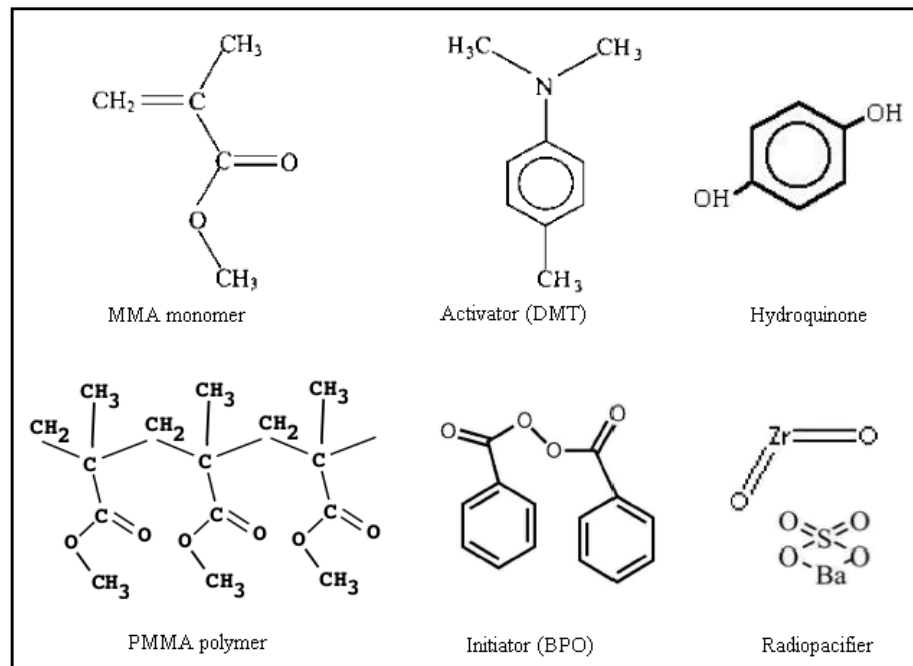


Figure 3: Main components of a typical acrylic cement

There are some advantages to using two bone cement components instead of simply polymerize pure MMA monomer: The polymerization of MMA is too slow compared with the duration of surgery. The monomer has a very low viscosity and can easily diffuse into the blood stream. It is much easier to shape the doughy cement to fill the space between the prosthesis and bone. The use of less monomer and the presence of pre-polymerized PMMA decrease the amount of released heat and assist in heat dissipation, thus lowering the overall temperature. Pure MMA, upon polymerization into PMMA, has a volumetric shrinkage of 21% due to differences in the density of the MMA monomer and the PMMA polymer. This contraction is unacceptable and would lead to a large gap at the cement-bone interface, compromising the fixation of the prosthesis [32].

Antibiotics

Surgical operating rooms have sterile conditions, but even under these conditions some bacteria can pass through all of the protective barriers and contaminate the open body tissues during the

surgery. In order to prevent post-operative infections, some small quantities of antibiotics can be added into the bone cement [11].

An acrylic bone cement is a meshwork of PMMA chains. Antibiotics enclosed in these meshes are released by elution from the bone cement. The elution properties of cements correlate directly with the ability to absorb water, which is determined by the hydrophobicity of their components [8].

The first trials of adding antibiotic a bone cement were performed in late 60s and the first antibiotic loaded bone cement appeared in 1970. Gentamicin sulphate was chosen due to its wide spectrum antimicrobial activity, water solubility, thermal stability, low allergenicity and ability to confer long-term protection [9]. Currently most of the 18 different antibiotic-loaded bone cements available on the market, contain gentamicin sulphate [33].

The prophylactic effect of gentamicin-containing bone cement on postoperative infections in total hip arthroplasties was compared with that of systemically given antibiotics [34]. It was observed that the incidence of postoperative infections in the patients with gentamicin-containing bone cement was significantly less than the group which was treated with systemic antibiotic therapy. It was also reported that the presence of small amounts of antibiotics did not change the handling characteristics and did not reduce the strength of the cement below acceptable standards.

The liberation of antibiotics from the cement matrix and the effect of antibiotics on the properties of the cement are two important issues [11]. Antibiotics are typically released in two stages: there is a peak release followed by a long tail of low level that continues for days or month. Approximately 90% of the drug may be retained inside the cement, being eluted only from the surface and from a network of cracks and voids in the bone cement by a dissolution–diffusion mechanism [35].

Several *in vitro* and *in vivo* studies have indicated bacterial growth on antibiotic-loaded bone cements with increased occurrence of gentamicin-resistant strains [36-38]. The increasing bacterial resistance to gentamicin has prompted renewed interest in the addition of further antibiotics to bone cements, such as tobramycin and cefuroxime [39, 40]. Multidrug targeting is assumed not only to be more powerful but also to prevent the emergence of resistant strains through the synergistic action of two antibiotics. In Europe, one multidrug-loaded bone cement containing gentamicin and clindamycin, Copal® is commercially available [33]. Combination of gentamicin and clindamycin in a bone cement formulation has a theoretical antimicrobial effect on more than 90% of the bacteria common to infected arthroplasties. The release of gentamicin seems to be enhanced by the release of clindamycin in this cement [41]. This may be an effect of the extra antibiotic, which acts as a soluble additive that leaves a network of voids behind, enhancing further release [42].

Multidrug targeting may be effective in preventing resistance but using it is a difficult option in bone cements, as the release of the different antibiotics depends on factors not easily controllable. For example vancomycin has a high molecular weight and shows poor release because it is trapped in the cement matrix [43]. Also, combinations of antibiotics must be carefully selected due to known cross-resistances. For this reason, it is not advisable to join the gentamicin and tobramycin. There is always the possibility of an antagonistic effect in the different ways by which the antibiotics act upon the bacterial life cycle [41].

PREPARATION OF BONE CEMENTS

Bone cements are prepared under operating room conditions, which consist of a temperature of 21-24 °C and a relative humidity no less than 50%. When bone cement was first introduced the only available method was hand mixing; in this case the powder-containing pouch is cut by a sterile scissor, and the contents are put in a sterile bowl. Then the liquid ampoule is opened, and the content poured on the powder. They are mixed at atmospheric pressure until a homogeneous dough is obtained (1–3 min) [11, 44, 45]. This type of mixing method can introduce a significant amount of air into the mixture and a relatively high degree of trapped porosity (5-16%) in the set cement [46]. To overcome these drawbacks, new mixing techniques have been introduced such as vacuum-mixing and centrifugation, aiming to reduce the porosity of the cement [45, 47]. Preparation of a cement by the hand mixing process and by a vacuum mixer are shown in Figure 4.



Figure 4: Cement preparation by hand mixing and by vacuum mixing.

Pores in the cement primarily result from air bubbles which have become entrapped during hand-mixing of the powder and liquid components, but it is also accepted that monomer evaporation at the high polymerization temperatures may contribute to produce embedded bubbles [48].

In vacuum-mixing, the bone cement is mixed while under a vacuum; which is supposed to eliminate the voids entrapped during the mixing process [49]. In centrifugation mixing the dough is immediately poured into a syringe that is then promptly placed in a centrifuge and spun with a speed of 2300 to 4000 rpm for 30-180 s [50], forcing out the air bubbles due to centrifugal forces [51].

After mixing, the cement is either manually placed into the cavity by means of “finger packing” or injected with a cement gun. The cement may also be pressurized at this time causing increased cement-bone interdigitation and providing a stronger interface [52].

CHEMICAL REACTIONS AND SETTING PROCESS

Mixing the two components (solid and liquid) produces the starting up of a typical addition polymerization reaction of the liquid monomer [7]. The MMA monomer can be polymerized through radicals formed by several different methods including the collision of two monomer molecules of sufficient energy or the decomposition of an initiator molecule by means of heat, light or chemical reaction [11, 53]. Bone cement polymerization is based on the free radical polymerization of MMA initiated by a redox system generated by reaction between the initiator (BPO) and the activator (DMT) which comprises three steps: initiation, propagation and termination.

The initiation step involves a reaction between the initiator and the activator causing the decomposition of the BPO, which splits into two fragments upon dissociation of the weak peroxy bond resulting in benzoyl radicals at room temperature. The second step is chain propagation, which basically consists in successive addition of monomer units to the active radicals already produced in the initiation stage. The free radical attacks one of the double bonds of the MMA monomer resulting in a larger free radical, then this new free radical attacks another MMA monomer and the chain propagates until a PMMA of relatively high molecular weight is formed. A consequence of this propagation phase is an increase in viscosity, due to the increasing concentration of polymer molecules and increasing molecular weight of the growing chains. Lastly, the chain termination can be achieved by combination of two chains (combination) or by hydrogen transfer reaction (disproportionation). The first method is the simplest way, wherein the two unpaired electrons join to form a bond [11, 32, 54].

When polymerization of the monomer is complete the pre-polymerized PMMA beads that form the powder (as described in "Typical compositions") are embedded into a solid PMMA matrix. The polymerization process is an exothermic reaction; in which heat is generated firstly when the benzoyl peroxide molecule is split, and secondly during the propagation stage of the reaction. The polymerization is very rapid and reaches completion in approximately 10–15 min, at which point the cement has set [55].

The variation of temperature with time, during the preparation of the material, can be monitored leading to a typical curve which indicates the setting process of the cement, Figure 5.

The time at which the mixed cement mass does not adhere to a surgically gloved finger is known as the *dough time*. This time is limited by the Standard Specification ISO5833 for acrylic resin cements [56] to a maximum of 5 minutes. The time elapsed from the moment at which the powder and liquid components are mixed until the cement is set, is known as the *setting time*. Setting time can be calculated as the time at which the temperature of the mass is the sum of the room temperature and maximum temperature divided by two. The ISO5833 establishes that the range time of the setting must be 5-15 minutes. The *maximum temperature* or peak temperature is produced by the exothermic propagation reactions which take place during polymerization. The cement sets before the peak temperature is reached. This value is limited to 90 °C. Finally, the difference between the setting time and the dough time is called the *working time*, and it corresponds to the period of time during which the cement is workable and has to be implanted (or molded) [3, 7, 57].

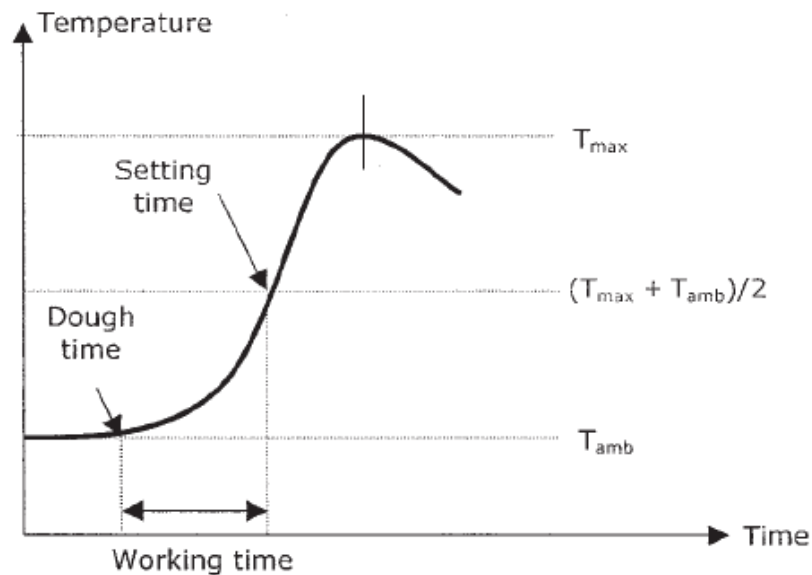


Figure 5: Temperature versus time curve of a curing acrylic bone cement.

The peak temperature recorded in vitro does not correspond to those actually reached in vivo. Clinical tests showed significantly lower intraoperative peaks at the bone-cement interface due to the thin layer of cement, the heat dissipation of the system via the implants and local blood circulation. The peak temperature in vivo is usually below the protein coagulation temperature, assumed to be around 56 °C [4, 7]. Meyer et al. [58] found that the maximum temperature of the curing bone cement could be lowered by reducing the ambient temperature and the cement mantle thickness.

The setting time is sensitive to ambient temperature, thus, when the temperature of the operating room increases, the polymerization rate also increases and the dough hardens quicker [11]. For example, the bone cement Surgical Simplex® P has a setting time of 9 minutes at room temperature of 24 °C, 12 minutes at 21 °C and 15 minutes at 18 °C [59]. Also the temperature of the powder and liquid components, of the implant and of the mixing equipment can markedly affect the setting time and the curing temperature. If the cement components are stored at temperatures lower or higher than that of the room, sufficient time must be allowed for them to reach the appropriate ambient operating room temperature before they are mixed, otherwise setting time will be correspondingly lengthened or shortened [60].

Others factors that can affect the curing properties of PMMA bone cements are:

The powder/liquid ratio of the cement, which is taken as the ratio between the weight of the powder in g and the volume of the liquid in ml, has a very strong effect on the curing parameters. Increasing the powder/liquid ratio (by increasing the amount of PMMA powder or by decreasing the content of monomer), produces the decrease of the peak temperature [58, 61, 62]. These results can be understood in terms of the relative amounts of monomer present whose polymerization causes heat release and of the role played by PMMA beads (powder) that absorb heat [7]. The optimal ratio given is about 2:1 (w/v), which is used in most of the commercial bone cement formulations.

PMMA bead size, i.e. the average diameter and size distribution of PMMA beads also plays an important role in the curing properties. Besides its structural role as a component of the cement matrix, PMMA beads serve as a heat sink, dissipating energy released by the exothermic polymerization of MMA. The incorporation of PMMA beads with larger mean diameters and widespread distributions of particle size has been reported to decrease the maximum temperature and delay the curing process [63, 64]. Pascual B. et al. [64] prepared formulations with different sizes of PMMA particles and their results indicated that the use of PMMA particles of 50-60 µm average diameter and size distribution of 10-140 µm reduced the peak temperature by about 30 °C and increased the setting time by 5-6 min, in comparison with commercial systems CMW®

(diameter 21 μm and interval 5-50 μm) and Rostal[®] (diameter 31 μm and interval 10-60 μm), without any noticeable mechanical deterioration.

Initiator and activator, the rate of radical formation is dependent on the concentrations of activator and initiator, being also necessary to take into consideration their effects on the setting parameters. Regarding the kinetics, increasing the amount of DMT and BPO increases the rate of polymerization and, consequently, the magnitude of the maximum polymerization temperature [64, 65]. Faster radical formation activates more monomers that act as nucleation sites for polymer chain growth and produces additional downstream effects such as: 1) acceleration of the overall polymerization process, decreasing setting time; 2) simultaneously formation of more individual polymer chains, decreasing the average molecular weight and affecting the mechanical properties of the cement. Vazquez et al. [66] reported that the peak temperature decreased with decreasing BPO concentration. The authors found that the difference in peak temperature for the formulation prepared with the highest concentration of BPO and the one prepared with the lowest concentration was approximately 10 °C and the setting time increased with decreasing initiator concentration, with differences around 5 min.

RESIDUAL MONOMER

Although most of the monomer in bone cement polymerizes, there is a small portion that volatilizes and escapes from the surface during polymerization. Another portion of that monomer becomes entrapped in the polymeric matrix as residual monomer [67].

During the curing of the cement a substantial increase in the viscosity of the mixture takes place due to a partial dissolution of the PMMA in its monomer. The polymer chains from the PMMA become available for free radical polymerization and entanglements of these chains with newly formed ones occurs, leading to an intimate connection in the structure [9].

The mobility of the monomer is greatly hindered by the increase in viscosity, and thus polymerization process evolves with difficulties stopping after a certain time without consuming all the present MMA monomer. In the curing process even the maximum temperature attained is lower than the PMMA glass transition temperature ($T_g = 100\text{-}120\text{ }^\circ\text{C}$) which hinders the total conversion of monomer into polymer [57].

As a consequence of the increase in viscosity of the cement, there is always an amount of 2–6% of non-reacted monomer that remains entrapped in the cement matrix after setting due to the decrease of free radicals diffusion rate [4].

Unreacted MMA not only acts as a plasticizer, influencing the mechanical properties of the cement but also leaks from the cement mantle into the surrounding tissues, causing toxic effects and impairing bone remodeling [68].

MECHANICAL PROPERTIES

The bone cement mechanical properties are very important in terms of clinical success and they have been studied in great detail and reported by many authors in several reviews [18, 69-73].

The function of bone cement is to fill the free space between the prosthesis and the bone. In this application it acts as an intermediary phase, fixing the implant to the bone, transmitting the applied force and body weight uniformly to the tissue and functioning as a load-bearing material [74]. If the transferred stress is higher than the capacity of load distribution, the cement can be fractured and the prosthesis can fail [75]. It is therefore very important that the cement is able to maintain its mechanical properties over a long period of time in vivo.

Static mechanical properties such as compression, tensile, flexural and shear are the relevant parameters to be evaluated in terms of the biomedical applications [57]. The variation of these properties is related to differences in composition, mixing methods, aging, temperature and viscosity during cement application [32]. It is known that acrylic polymers are stronger in compression than in tensile, and exhibit a viscoelastic behaviour, which means that their properties strongly depend on temperature and strain rate [22].

The addition of radiopaque agents has a significant effect on the mechanical properties of acrylic bone cements, which depend on their size and morphology [7]. The presence of antibiotics diminishes its mechanical properties, although the reduction depends markedly on the amount of antibiotic added [76].

An important factor that affects the bone cement mechanical properties is the porosity of the samples. Pores can act as weak points concentrating tensions and initiating a fracture [57]. These pores may be attributed to air entrapped during mixing, monomer evaporation over polymerization and shrinkage around particles, giving rise to formulations that can have 2-10% pores volume fractions [77]. To reduce its formation new mixing techniques have been introduced such as vacuum-mixing and centrifugation.

The influence of body fluids and body temperature (37 °C) can be relevant to different properties of the cement. Sorption of water generally lowers the mechanical properties [7]. However, fracture mechanics studies show that the crack velocity is slower in water than in air, and that fracture toughness is about 15 to 20% higher in water than in air [78].

Low viscosity cements might not withstand the bleeding pressure in the femur with the consequence of blood entrapment within the cement representing potential areas of weakness with increased fracture risk. Normal or high viscosity cements in this regard seem to be more appropriate resulting in better long-term performance [3, 4]. High-viscosity bone cements have shown to offer a lower incidence of revision and aseptic loosening in total hip arthroplasties [11].

It has been reported that microcracks are usually developed in the interbead matrix just before failure and not through the pre-polymerized beads. These cracks propagate, and gross mechanical failure occurs [79]. The failure can also begin due to cracking induced by residual stress around pores or stress raisers. Some residual stresses are caused by the temperature differences arising after polymerization of bone cement [80].

ANCHORAGE MECHANISMS

The interfaces cement-bone and cement-prosthesis are considered the weak-link-zones in total hip arthroplasty [18]. Bone cements do not form chemical bonds either with the metallic implant or with the natural bone. They fix the prosthesis in the desired area by forming a mechanical interlock between the metallic implant and the bone, and transfer the load from one to the other. Bone cement diffuses into the microscopic irregularities of the bone cavity and provides a mechanical attachment to bone (interdigitation) [11].

The strength of the cement–bone interface and the success of an implant are related to the amount of interdigitation between the cement and the cancellous bone [81]. Cement pressurization improves cement intrusion into bone and this phenomenon may improve fixation, although it has been reported that it may also increase bone resorption and reduce bone formation [82]. The cement should be pressurized as early as possible within the rasped cavity (immediately after the dough stage if possible) [83]. The apparent strength of the cement–bone interface is significantly higher when the interface is loaded in shear rather than tensile loading [84].

The cement-implant interface is not very strong, similarly to what occurs with cement-bone. The attachment of bone cement to metallic implant is generally achieved by selecting an implant surface texture that creates a mechanical interlock with the cement or by an implant with a geometry that maintains stability. Higher surface roughness of the prosthesis leads to better fixation, since it allows for increased surface area contact with the cement as well as deeper interdigitation [85]. The interfacial bond strength also depends on the material of the prosthesis [86].

The cement-implant interface can be improved by pre-coating the prosthesis with bone cement or poly(methyl methacrylate) polymer. During the surgery, the fresh cement adheres well to the

pre-coating cement [87, 88]. It was reported that PMMA coating increased the torsional fatigue strength of the metal–cement interface [89].

MAIN DRAWBACKS OF COMMERCIAL BONE CEMENTS

Aseptic Loosening

Aseptic loosening occurs when the implants become loose within the bone; a loose implant tends to be painful and frequently requires a revision surgery. Aseptic loosening is the main cause of failure of cemented total hip arthroplasties, being often associated with significant bone resorption, necessitating the use of special prostheses and bone grafting [90, 91]. During surgical revision of a loose cemented implant, a characteristic fibrous membrane is identified at the interface between the bone and the bone cement [92]. This fibrous membrane is laden with histiocytes and giant cells surrounding and engulfing cement, polyethylene and metallic debris [91].

The release of particles by the cement or by the prosthetic components can precede the mechanical instability and be the cause of loosening. It was shown that monocytes and macrophages responding to particles of bone cement are capable of differentiating into osteoclastic cells that resorb bone. Usually it is observed that no bone trabecula reaches the cement surface due to the presence of fibrous tissue [93]. This membrane can be caused by the toxicity of monomer release and the heat production of the polymerization, resulting in instability and movement at the interfaces [94]. These micromovements at the bone–cement and stem–cement interfaces can accelerate aseptic loosening.

Failure of PMMA increases bone resorption at the bone–cement interface of the prostheses. When this happens, new particles which are small enough to be phagocytized are produced. Phagocytosis of the particles results in the increased production of tumor necrosis factor by the macrophages, which may in turn lead to bone resorption and prosthetic loosening [95].

Loosening of the cemented prostheses involves not only the failure of the implant and/or the bone cement, but also the inflammatory response of the bone tissue against bone cement components. For example, it was shown that the inflammatory response to PMMA particles containing BaSO₄ was greater than the response to plain PMMA particles of similar size [96].

The main factors involved in aseptic loosening and periprosthetic osteolysis are summarized below [97]:

Wear debris induced osteolysis: integration of the prosthesis into the surrounding bone can be hindered by a “foreign body reaction” induced by macrophages absorbing small particles, mainly

polyethylene, PMMA and metallic debris, leading to activation of osteoclastic activity. As a consequence, osteolysis and bone loss around the implant occur.

Micromovement of surfaces: implants that do not achieve adequate initial fixation will exhibit micromotion in response to load. The greater the area of friction the more osteoclasts are activated causing osteolysis around the implant which leads to fatigue failure at interfaces. When the distance between bone and implant exceeds 150 μm , connective tissue membranes are formed between implant and bone as well as between implant and cement. These membranes hinder the osteo-integration of the prosthesis.

Inappropriate mechanical load and stress shielding: insertion of an implant leads to new biomechanical relationships between various regions of the surrounding bone and the implant. As a consequence of stress shielding, bone apposition and higher bone density occur in regions around the implant receiving high loads, whereas regions receiving lower stress loading react with bone loss. Appropriate load transmission is thus an essential factor in maintaining bone volume. Optimal load transfer is influenced by the design and stiffness of the implant.

Post-operative immobilization: the post-operative decrease in weight bearing results in local immobilization osteoporosis. Overall the post-operative bone loss mainly occurs in the first 6 months and can reach up to 50 % of the former bone stock.

Operative trauma: thermal and mechanical necrosis caused by surgical procedure, type of bone cement and cementing techniques can alter bone quality.

High Polymerization Temperature

One of the main side effects of acrylic bone cements application is the rise of the temperature at the bone–cement interface during the polymerization of MMA. In bone cement formulations the powder part is already made of pre-polymerized PMMA particles, and this prevents the explosive polymerization reactions [11]. The highly exothermic polymerization process of the MMA, with a polymerization heat of 57 kJ per mole MMA, causes an increase of the local temperature [4]. The peak of temperature can vary from 80 to 124 °C [11, 98].

According to the ISO5833 [56], standard for acrylic bone cements, the maximum temperature allowed in the setting reaction must be lower than 90 °C (recorded using a device at room temperature). The levels for thermal tissue damages in bone are estimated to be between 48 and 60 °C, and within this temperature range cell necrosis also depends on the exposure time [99]. In clinical hip or knee replacement, maximal interface temperatures as low as 48 °C and as high as 105 °C have been reported [100].

In some cases bone cements originating high temperatures may be desirable. Some surgeons treat the giant cell tumors of bone tissue by using the technique of aggressive curettage through a

large bone window followed by acrylic cement reconstruction [101]. As the bone cement self-heats, the possibility of heat necrosis in the bone tissue exists. It was mentioned that the damage to the cells due to heat may be beneficial in reducing the rate of tumor recurrence [102].

Release of MMA monomer

As most of the organic monomeric chemicals, MMA itself is also toxic to the bone tissue. The release of MMA monomers from the cement into the circulating blood causes severe drop in blood pressure leading to an increase in the heart rate and impairing bone remodeling [11, 94]. This is caused by the direct chemical effect of MMA on blood vessels. The presence of MMA has been also associated with irritation of skin, eyes, and mucous membranes, allergic dermatitis, liver toxicity, fertility disturbances, arterial oxygen tension and possible cardiac arrest [103].

The proportion of residual monomer remaining in the polymerized bone cement is in the range of 2–6% just after hardening [4]. This percentage may decrease by up to 1-2% with time and then remain the same for years. Haas et al [61] measured the residual MMA monomer content to be 3.3% after 1h, 2.7% after 24h and 2.4% after 215 days under storage in an ambient air environment. Schoenfeld [104] found that most of the methyl methacrylate is released in the first hour and its toxicity disappears after 4 hours.

ALTERNATIVES TO THE STANDARD COMPONENTS

Radiopaque Agents

PMMA is not a radiopaque material, i.e., it is almost impossible to determine the borders of the cement applied during the surgery by ordinary x-ray imaging. Since 1972, radiopaque materials have been added to the bone cement in order to provide radio-opacity [11]. Addition of about 8-13% of barium sulfate (BaSO_4) and 9-15% of zirconium oxide (ZrO_2) to the powder part confers higher opacity. Otherwise, the areas occupied by bone cement can be determined by using magnetic resonance imaging [105]. Additional opacifiers are often used for interventional procedures such as vertebroplasty, in which visibility is a key issue.

The presence of radiopaque materials may have some disadvantages. The lack of interaction between filler and matrix is the main reason for the detrimental effect of these particles on some mechanical properties and to the liberation of particles into the surrounding tissue [106, 107]. It was also observed that, osteolysis, i.e., bone resorption around bone cement application area, was more severe when radiopaque agents were used [108]. This situation was more evident for BaSO_4 than for ZrO_2 case [109, 110].

The development of radiopaque agents miscible with the polymer matrix as alternative routes for achieving radiopacity is an area of interest in bone cement field. The possibility to confer radiopacity by introducing an x-ray opaque iodine containing methacrylate in the liquid phase of the bone cement has been studied and 2,5-diiodo-8-quinolyl methacrylate (IHQM) was proposed as a new radiopaque agent. It was reported that the incorporation of IHQM yielded a decrease in the peak temperatures and a slight increase in the setting time. A content of 2 wt% of IHQM (over the total mass) was enough to render the cement radiopaque with acceptable values of curing parameters and enhanced mechanical properties [111]. IHQM provided significant improvements in tensile strength, toughness and ductility when comparing to both ZrO_2 and BaSO_4 containing cements [106, 112]. The improvement of mechanical properties was due to both, the elimination of porosity associated to the BaSO_4 particles and the reinforcing effect attributed to the iodine-containing monomer [112].

The 4-iodophenol methacrylate (IPMA) was another compound synthesized to confer radiopacity, via in situ polymerization. Having a higher molecular weight than MMA, it leads to a decrease in the monomer concentration, resulting in shorter polymerization time, although T_{max} was approximately constant. A content of 15% IPMA conferred radiopacity equivalent to 10% BaSO_4 . Regarding mechanical properties, the performance of the formulation with IPMA was better than that with BaSO_4 [113, 114].

Organo-bismuth compounds such as triphenyl bismuth - TPB (a heavy metal containing organic compound which is relatively non-polar and thus hydrophobic or insensitive to moisture) were also studied as radiopaque agents by Deb et al. [107]. It was found that addition of TPB to the bone cement matrix up to 25% of the weight of the polymer did not affect the polymerization temperature and setting time. Performing the addition via dissolution in monomer phase, an increase in strain and reduction in brittleness was observed. The best mechanical properties were obtained for 10% TPB in solution.

More recently, two bromine containing monomers, 2-(2-bromoisobutyryloxy)ethyl methacrylate (BIEM) and 2-(2-bromopropionyloxy) ethyl methacrylate (BPEM), were synthesized and characterized as being good candidates to be used as radiopacifiers [115]. The addition of BPEM decreased the maximum temperature and increased the setting time, when compared with the radiolucent cement. It also decreased the glass transition temperature, enhanced the thermal stability, reduced the polymerization shrinkage and increased the compressive strength of the resultant material [116].

Activators

Tertiary aromatic amines are currently used as activators in the curing of acrylic bone cements. Commercially available acrylic bone cements usually contain N,N-dimethyl-4-toluidine (DMT) in a range of 1.5–2.5 wt.% as an activator in the polymerization of the MMA monomer initiated by benzoyl peroxide (BPO) [117]. Figure 6 illustrates the chemical structure of some tertiary aromatic amines used in the curing of acrylic resins [118].

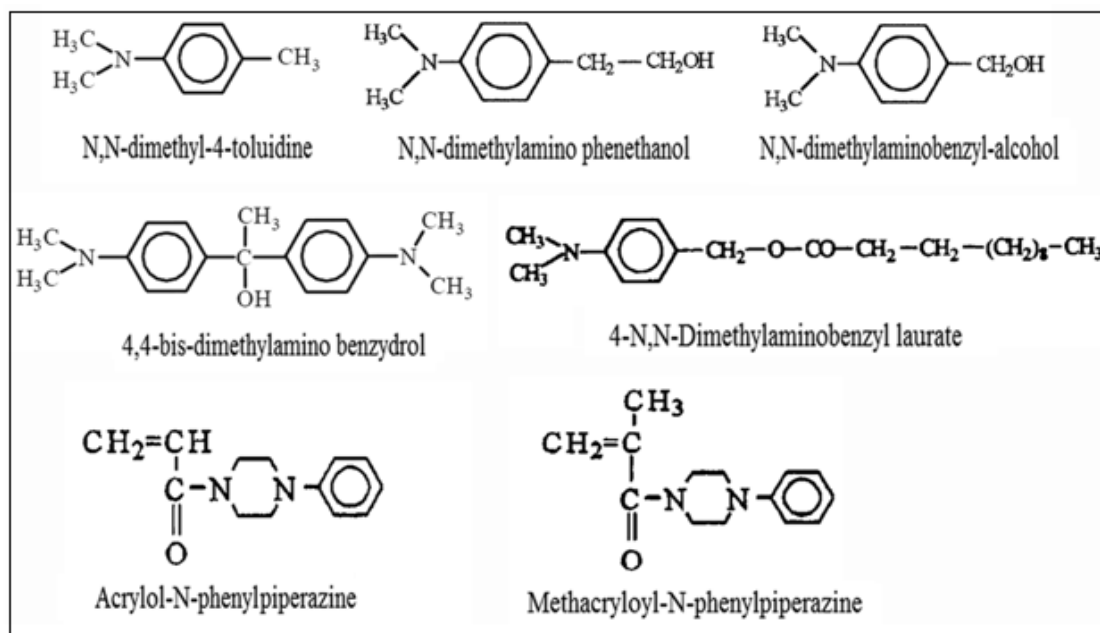


Figure 6: Chemical structure of activators used in MMA polymerization.

Unreacted DMT may be present in small quantities in cured cements (0.5-0.7%) even after long-term storage in air or post-implantation [119]. The toxicity of the amine/BPO initiation system is related to the mobility of the amine. The tertiary aromatic amines are low molecular-weight compounds, which may easily leach out from the acrylic cement to the surrounding tissues [120].

In vitro studies in osteoblasts culture demonstrated that DMT causes a delay in the cell replication, induces chromosomal alterations and inhibits protein synthesis, interfering with the process of bone mineralization [118, 121]

Few commercial bone cement formulations contain an activator different from DMT. The Sulfix-60® cement, which has incorporated a tertiary aromatic amine of reduced toxicity (N,N-dimethylamino phenethanol), when tested in vitro with osteoblast-like cells, produced the most positive response among other commercial formulations [121, 122]. However, with respect to in vivo studies scarce references are found in the literature.

The application of some tertiary aromatic amines with reduced toxicity to the curing process of acrylic bone cements has been recently studied with the aim of obtaining cured materials with

improved biocompatibility [123]. In this study the authors analysed and compared the properties and preliminary in vivo response to acrylic bone cements cured with N,N-dimethylaminobenzyl-alcohol (DMOH) and with 4,4-bis-dimethylamino benzydrol (BZN). Both activators presented LD₅₀ (lethal dose, 50%) values 3-4 times higher than DMT, were less cytotoxic against polymorphonuclear leucocytes and possessed antimicrobia character. The cement formulated with the activator BZN gave the most promising response and the best biocompatibility: rests of connective tissue were found attached to the material after intramuscular implantation and higher and earlier osseous neoformation were observed after intraosseous implantation.

4-N,N-Dimethylaminobenzyl laurate (DML) is a tertiary amine activator with longer alkyl chain, possessing therefore a hydrophobic nature and higher molecular weight in comparison to DMT, being consequently less prone to leaching than DMT [120, 124]. It could as well initiate polymerization by amino methyl radicals, with the incorporation of DML into the macromolecular chains. It leads to longer t_{set} , due to the longer diffusion time of the amine and to its long hydrophobic chain [120]. Although the pure DML decreased cell viability and cellular proliferation compared to the control, the cured cements showed good biocompatibility, with cells adhering and proliferating on the test materials and exhibiting normal metabolism and morphology [124].

Tanzi et al. [125] investigated the substitution of DMT by unsaturated tertiary arylamines, such as acryloyl- (ANP) and methacryloyl- (MNP) N-phenylpiperazine, considered as being less toxic. Compression tests revealed that compressive yield stress, strain at yield, and elastic modulus values were quite similar to those of samples cured with DMT and ANP, and slightly lower results were obtained with samples cured with MNP. This work has shown that it is possible to use alternative tertiary amines with reduced toxicity as activators without significant changes in the curing behaviour and properties of the acrylic resins.

BIOACTIVE BONE CEMENTS

One of the major limitations of commercially available acrylic bone cements is the lack of adhesion to bone, which may cause aseptic loosening and failure of the prosthesis in some cases. Since there is no chemical adhesion to the bone and no bone growth stimulation the only source of bonding/adhesion of conventional bone cements is the mechanical interlocking with bone interstices and this process is not enough to assure the stability of the system [11]. In reality due to the exothermic effect of the polymerization reaction and the toxicity of the MMA, bone necrosis can occur, together with the formation of fibrous tissue around the implant which allows micromotion and the lack of fixation stability at the bone-implant interface, causing pain to the patient and a space for the accumulation of wear particles [94, 126]. Thus, in an effort to enhance

prosthesis integration and to prolong the lifetime of the implant, researches have been carried out on many different types of cements.

Aiming to promote the bioactive behaviour of a bone cement, three types of formulations have been suggested [126, 127]:

- 1) An all-bioactive bone cement where the whole material is bioactive, for example calcium phosphate cements.
- 2) A surface-bioactive cement where bioactive filler particles are added to a non-bioactive matrix, for example HA or bioactive glass added to PMMA cement.
- 3) An interface-bioactive bone cement where a bioactive material is placed between the bone and the non-bioactive cement, for example a layer of HA granules between bone and cement.

Recent studies are showing that the second possibility, i.e. addition of bioactive fillers, seems to be the most promising procedure to improve the interfacial strength of cement to the bone. These fillers will be exposed at the cement-bone interface, promoting the direct growth of bone towards the cement surface and thus increasing the interfacial strength. The most investigated fillers are hydroxyapatite (HA), glasses, glass-ceramics and tricalcium phosphate (TCP) [94].

Besides providing the bioactive behaviour, such fillers may also contribute for enhanced mechanical properties. However the amount of filler particles in the cement is of major importance since it can produce the deterioration of the mechanical properties for high contents or the lack of bioactivity for low contents [126].

Bioactive cements filled with calcium phosphates

A large number of studies has been carried out on the addition of hydroxyapatite (HA) into acrylic bone cement in order to improve mechanical strength as well as to enhance biocompatibility. Calcium phosphates can replicate the structure and composition of bone minerals in a reproducible way, so they have a biocompatible behaviour with bone cells [128, 129]. Furthermore HA discloses osteoconductive properties allowing the formation of bone on its surface by attachment, migration, proliferation and differentiation of bone forming cells [130].

One of the first reports on the addition of HA to bone cements was published in 1983 by Giunti et al. [131]. The authors varied the percentage of HA from 0 to 50%, and observed enhanced mechanical properties as well as a decrease in curing temperature. The heat generated during PMMA polymerization may become significantly attenuated when HA is added [132], reducing the problems associated with cell death due to high polymerization temperatures.

The results on the effect of calcium phosphates on the mechanical properties of bone cements are not always concordant and clear and they depend on a number of factors such as type of filler,

filler concentration and matrix chemical composition, among others. These factors also affect the curing characteristics of the cements.

Alterations in the HA concentration produce changes in the mechanical behaviour of the cements because these particles usually act as porosity condensation centers thus modulating the porosity patterns of the cements [133-135]. It is reported that porosity and pore size usually increase with increasing fraction of filler up to a certain limit that determines the further impoverishment of the mechanical properties [135]. A maximum value of the Young's modulus (2.5 - 3.5 GPa) and of the flexural strength (58 to 69 MPa) was obtained by Olmi et al [134] for an HA proportion of 3 wt%, corresponding to a minimum porosity, but other authors [135] found flexural modulus and fracture toughness values limited to a maximum of 15 % (w/w) filler. These authors also verified that the compressive yield strength of samples containing 2.5 % of HA was higher than that of the unreinforced cement, but lower for higher HA percentages due to the degree of adhesion between the HA particles/matrix and the formation of pores giving rise to a weak interface. Considering creep properties an improvement in creep resistance was achieved with up to 5 wt% of HA [126].

Since the addition of HA into the formulation increases the viscosity of the cement dough and makes handling and workability difficult, very low viscosity (VLV) cement compositions were developed, in order to achieve homogeneous distribution of HA particles [136]. Acrylic bone cements filled with HA demonstrated higher mechanical strength than the reference cement (commercially available CMW1 bone cement). The addition of HA into VLV cement compositions provided a decrease in curing temperature, an increase in compressive strength and compressive elastic modulus, and a slightly increase in terms of fatigue strength and fatigue life compared to CMW1. On the other hand, it was observed that this cement is weaker in tension tests than the reference.

The HA effect in the mechanical properties of the bone cement depends on the amount, as mentioned above, particle size and surface properties of the particles. The introduction of HA to commercial formulations varying the particle size (2 to 137 μm) and HA content (2 to 25 wt%) produced great changes in the mechanical properties of the composites [126, 137]. Static tests revealed that up to 10 wt% of HA with particle size of 96 μm could be added without any large decreases in tensile strength. Fatigue results also showed that adding 10 wt% of HA of different average size had no significant effect or actually increased the fatigue resistance of the cement. However, when the concentration of filler was increased from 10% to 20% a tendency to decrease the tensile strength was observed for all particle size.

The interface between HA and polymer matrix plays a critical role in determining the mechanical properties of the cements and the lack of adhesion between the two phases can result in

an early failure. The linkage of the organic hydroxyethylmethacrylate (HEMA) to apatitic calcium phosphate can be realized by a combination of thermally stimulated current and dielectric spectroscopy. The obtained apatite is used to form chemical bonds with the polymer matrix, which could stiffen the PMMA bone cement [138]. An improvement in the mechanical properties without affecting the biocompatibility of the HA-containing bone cement was also obtained through the addition 4-methacryloxyethyl trimellitate anhydride (4-META) into MMA monomer as an adhesion promoting agent [139]. However, it is reported [140] that in a P(MMA-co-styrene)/MMA acrylic cement filled with HA particles coated with a silane agent, all investigated mechanical properties except tensile modulus had lower values for HA-filled cement compared to the unfilled cement. The results were attributed to HA powder agglomeration and to the formation of porosity.

The water absorption properties of a bone cement are critical to its long-term stability *in vivo*, since they can lead to a reduction in the strength of the polymer. The water absorption characteristics of modified HA-reinforced poly(ethyl methacrylate-*n*-butyl methacrylate) (PEMA_nBMA) bone cements were assessed [141]. The introduction of HA reduced the water uptake, yielding more significant results if the HA filler was surface treated with a silane coupling agent.

Among the HA filler advantages, we find cements with lower curing temperature and residual monomer content when compared with those for unfilled cements [140, 142], and better biocompatibility, with less severe necrosis and foreign body giant cell observed for this cement [136]. The effects of the incorporation of only 6 vol% of HA into PMMA provided higher levels of human osteoblast-like cells (HOB) proliferation and phenotype expression. Exposed HA particles served as preferential anchoring of HOB cells. Although both conventional and composite bone cements were able to support normal osteoblast cell growth, full confluence was achieved earlier (7 days) on the PMMA-HA cement [143, 144].

A comparative study of the osteoblastic response on a PMMA/HA (80/20 wt%) composite and on a non-filled PMMA was carried out by Moursi et al [145]. Osteoblast attachment and proliferation were similar on both implant materials for 2 days, whereas, on day 8 proliferation was significantly higher on PMMA/HA than on PMMA. Compared to PMMA, PMMA/HA composite promoted the formation of nodules displaying a higher degree of mineralization, considered the strongest indicator of true osteoblast differentiation and osteogenesis.

In vivo studies in rabbits with implantation of HA-filled cements [146] also revealed a noticeable increase of attachment to bone tissue for the higher HA specimens improving the interfacial shear strength at the bone-implant interface six weeks after implantation into the distal end of rabbit femora.

Although at a lesser extent than HA or bioactive glasses and glass-ceramics α -TCP, has also been studied as filler in bone cements. α -TCP is highly soluble at physiological pH and can originate a porous structure capable of osteointegration. It was demonstrated that the curing temperature of the bone cement can decrease from 100 °C to 58 °C by adding 66% of α -TCP [147]. According to Yang [148], when α -TCP was incorporated into commercial formulations it retarded the polymerization kinetics and decreased the heat release rate. This effect would decrease the thermal necrosis of bone and also improved the thermal stability of the system.

Osteoblast cultures (MG63 line) were tested with PMMA/ α -TCP composites [149] and it was demonstrated that PMMA/ α -TCP significantly and positively affected osteoblast viability as compared to PMMA. At 12 weeks, the PMMA/ α -TCP implants in rabbit bone successfully osteointegrated in trabecular and cortical tissue. The presence of the bioactive ceramic material showed to be responsible for the improvement of: the material colonization by bone cells, osteoblast activity, osteoinduction and osteoconduction processes, and bone remodelling.

Bioactive cements filled with glasses

The first bioactive glass studied was Bioglass® 45S5, introduced by Hench in 1971 [150], which still remains the most used in clinical applications and the most promising one. Bioglass® 45S5 is produced by melting, and its specific composition is 45% SiO₂, 24.5% CaO, 24.5% Na₂O, 6% P₂O₅ (expressed as weight %). The name '45S5' refers to both the SiO₂ content (45% wt) and to the Ca/P molar ratio (5).

In fact, when these glasses are put in contact with biological fluids, a layer of hydroxyapatite (HA) analogue to the mineral phase of bones is deposited on their surface. Collagen molecules are incorporated into this layer, and a biological bond can be formed. It was later shown that a bond with soft tissue can be achieved as well, if the rate of apatite formation is high enough [151].

Five inorganic reaction stages (Figure 7) occur at the glass surface when a glass is immersed in a physiological environment [152]:

1. Ion exchange in which modifier cations (Na⁺, Mg⁺², Ca⁺²) in the glass exchange with hydronium ions (H₃O⁺) in the external solution;
2. Hydrolysis in which Si-O-Si bridges are broken, forming Si-OH silanol groups, and the disruption of the glass network;
3. Condensation of silanols to form a silica gel layer;
4. Precipitation of Ca and phosphate on the gel, leading to the formation of amorphous calcium phosphate;
5. Gradual transformation of calcium phosphate layer into crystalline hydroxyapatite

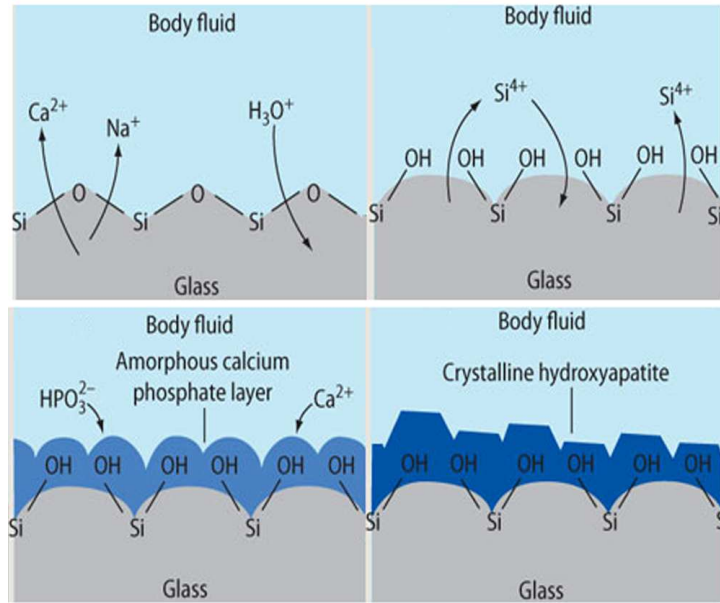


Figure 7: Mechanism of apatite formation on glass adapted from [153] .

Several glass compositions may promote bioactivity and they can be further explored and used to improve bone bonding capability of inert matrices like PMMA. Compared to synthetic hydroxyapatite, the surface layer formed on bioactive glass is more similar, in terms of crystallinity, to the apatite of bone tissue, which produces a greater proportion of bone bonded to bioactive glass than to HA [154].

The addition of glass, glass-ceramic or ceramic particles to commercial cements affects their mechanical, curing and biological properties.

Flexural, compressive, and fracture properties of commercial acrylic bone cement modified by different weight fractions of glass spheres were altered by the filler content [155]. It was found that glass particles added up to 50 wt% produced significant increases in flexural modulus and fracture toughness, while contents higher than 25 wt% promoted a decrease in the compressive yield strength in cements cured at room temperature. The mechanical behaviour can be understood in terms of the reinforcing effect of the filler and the plasticizing effect of the monomer, which reduces the compressive strength and increases K_{IC} .

Recent researches, considering the bending properties, demonstrated that PMMA-co-EHA cements containing 30, 40 and 50 wt.% of glass of the $3\text{CaO} \cdot \text{P}_2\text{O}_5\text{-MgO-SiO}_2$ system, achieved a maximum flexural strength of 29 MPa coupled with an elastic modulus of 1.1 GPa at an intermediate filler concentration (30 wt.%), increasing its mechanical performance to the upper level of the values reported for cancellous bone [156]. For materials containing proportions of filler higher than 30 wt.%, the strength decreased steadily with increasing glass content due to the weak

bonding between the matrix and the filler, attaining similar values to those of the matrix material for 50 wt.% filler.

Cement formulations based on PMMA/phosphate glass (PG: 44.5% P_2O_5 ; 44.5% CaO; 11% Na_2O , mol%) [157] or PMMA/MgO-CaO-SiO₂-P₂O₅-CaF₂ glass (GBC: 4.6% MgO; 44.7% CaO; 34.0% SiO₂; 16.2% P₂O₅; 0.5% CaF₂, wt %) [158], in which different proportions between 30 and 70 wt% of glass were used, exhibited appropriated mechanical performance. It was verified that the elastic modulus of both cements increased with the increase of the filler content. A significant increase of compressive strength was obtained for the composites prepared with 20% (110 MPa) and 40 wt% PG (118 MPa), whereas for cement filled with higher PG contents the strength was equal to PMMA pure (100 MPa). For GBC cement, the compression and bending strength of all studied formulations were higher than those of conventional PMMA bone cement. The values of strength of PMMA were 74 MPa for compression and 92 MPa for bending, which increased for 131 MPa and 134 MPa respectively, when 70 wt% of glass was introduced (GBC70).

Together with the amount of added filler, the mean particle size and its amorphous/crystalline nature may also affect the mechanical behaviour of the PMMA-based composites. In bioactive bone cements consisting of PMMA and bioactive glass beads of the MgO-CaO-SiO₂-P₂O₅-CaF₂ system [159], was demonstrated that the bending strength increased as the mean size of the glass beads decreased (mean diameters 4, 5, 9, and 13 μm) due to improvement of filling effect, while Young's modulus seemed to be independent of particle size, i.e. it did not change significantly. Additionally, the smaller particles have larger surface area, this may help expose more bioactive filler on the surface of the cement, creating more contact with the bone. However, they may also cause strong foreign body reaction, thus care should be taken to avoid their separation from the bone cement surface. New cement formulations developed in the Kyoto University in 1993 [160] consisting of a bisphenol- α -glycidyl dimethacrylate-based resin (Bis-GMA-based resin) and a bioactive filler (glass or glass-ceramic) have shown values for compressive strengths that were lower for the composites filled with glass (153-180 MPa) than for the composites loaded with glass-ceramic particles (167-194 MPa).

Concerning the curing properties, values of dough, setting and working time usually increase with the content of glass in the cements. On the contrary, the maximum temperature reached during the polymerization reaction decreased with increasing concentration of glass [157]. The advantage of adding bioactive filler (glass beads) in PMMA, apart from bioactivity and better mechanical properties, is the decrease in maximum temperature of polymerization (T_{max}). It was observed that the temperature during polymerization was 68.3°C for the cement designed GBC and 87.5°C for the commercial PMMA bone cement (CMW-1®).

Bioactive and osteoconductive properties of acrylic bone cements with different types of filler are reported in the recent literature. Composites containing 30, 40 and 50 wt.% of glasses of the $3\text{CaO} \cdot \text{P}_2\text{O}_5\text{-MgO-SiO}_2$ system, exhibited in vitro formation of an apatite layer on the material surface, being faster for the higher glass contents [156]. Results obtained by Kobayashi et al [161] with cements containing bisphenol- α -glycidyl dimethacrylate-based resin (Bis-GMA-based resin) and a bioactive filler (glass ceramic powder AW-GC, HA or β -tricalcium phosphate (β -TCP) revealed that: after soaking in simulated body fluid (SBF) for 2 days, the AW-GC cement and the HA cement formed bonelike apatite over their entire surfaces but the β -TCP cement did not.

Osteoconductivity of several bone cements loaded with bioactive fillers was confirmed for different composite formulations [158,159,161-163]. Histological examination generally showed that a more effective contact with bone is facilitated by increasing the filler proportion. The osteoconductivity can also be evaluated by affinity indices (percentage). The calculation of affinity index is made from one SEM photograph and based on length of bone in direct contact with the cement surface divided by the total length of the cement surface, being this value multiplied by 100. It was demonstrated that the affinity index of the cement increases with a higher glass content and lower mean glass bead size [158,159]. An evaluation of interface between the bone and a bioactive glass cement revealed that the interfacial strength (push-out load of cylinders in contact with bone) of bioactive cements was much higher than that of CMW-1[®] [162] when implanted into canine femora. The interfacial strength values of the bioactive glass cements also increased with prolonged implantation time. Fujita and co-workers [163] examined the influence of the proportion (0, 30, 50, 70 and 80 %) of AW-GC glass-ceramic powder on the bone-bonding ability of Bis-GMA-based cement. The developed cements were implanted into the proximal metaphysis of the tibiae of male rabbits, and the failure load was measured by detaching tests 10 and 25 weeks after implantation. The failure load increased with increasing content of glass and it was greater for 25 weeks. The results show that all the tested cements had the ability to bond to bone and to function as bioactive composites.

CEMENTS FILLED WITH BIODEGRADABLE FILLERS

The introduction of biodegradable fillers in acrylic bone cements has aimed the development of a system for controlling drug delivery, since it was discovered that the most of the antibiotic could remain inside the cement for many years. Scarce examples have been reported considering the bioactive behaviour of the cement associated to the strategy of bone growth inside the pores created during the degradation of the material, which could simultaneously facilitate bone replacement, ingrowth and bonding.

A comparative study between a conventional PMMA bone cement and a biodegradable cement based on PPF-MMA, in which a hydrolysable pre-polymer poly(propylene fumarate) (PPF) was cross-linked with MMA monomer, both carried with antibiotics and implanted subcutaneously in rats, revealed that the biodegradable cement PPF-MMA achieved and maintained considerably higher wound antibiotic levels than the PMMA cement [164].

Poly-L-lactic acid (PLLA) has been used to fill a PMMA matrix and to develop a drug delivery system [165-167]. The rate of ibuprofen release was analyzed and it was found to be affected by the crystalline or amorphous form of the drug. The incorporation of a ceramic component, Al_2O_3 , in this composite and the presence of the biodegradable polymer, PLLA, facilitated the ibuprofen crystallization and consequently its rate of release [165]. When a bioactive glass powder of the $\text{SiO}_2\text{-CaO-P}_2\text{O}_5$ system was added to the PMMA-based composites containing PLLA, [166, 167] it was observed the formation of an apatite-like layer in SBF although the crystallinity of this layer was lower on the composite than on the glass samples [166]. The rate of drug release was related to the ion exchange between the glass powders in the composite and the SBF. The loading of these composites with gentamicin led to a fast initial release during the first 10 h of soaking in SBF, followed by a controlled release of the drug. The results were similar for addition of ibuprofen, the growth of an apatite-like layer on the materials surface was demonstrated and the ibuprofen release rate was related with the growth kinetics of this layer, being slower when the materials do not contain the biodegradable polymer PLLA [167].

The presence of poly(ϵ -caprolactone) (PCL), in the partially biodegradable acrylic composites, provided a significant decrease in both compressive strength and elastic modulus when compared with the PMMA [168]. Composites loaded with 3% wt/wt vancomycin eluted 64% of the initial drug within the first 5 h, allowing a progressive release of nearly the total amount of the initial drug (90%) in approximately 2 months. The use of PCL beads as a solid component of the material provides lower peak temperature and longer setting times than the classical PMMA-based acrylic cements. The PCL/PMMA composites presented residual monomer values in the range of 2–3% wt/wt independently of the PCL content, and degradation test resulted in a weight loss close to 2% wt/wt and water uptake values in the range of 1.5–2%, after 56 days in SBF.

Literature also refers the incorporation of the biodegradable thermoplastic starches (TPSs) in self-curing acrylic cements aiming the achievement of a short- to medium-term drug delivery system for hard tissue treatment [19]. The cements exhibited values of water absorption up to 15.3% and mechanical properties in the range of accepted values according to standard specifications, although decreasing after immersion in PBS. The TPSs can gradually dissolve giving rise to a surface porosity, which induces a higher level of delivery of the drug (Ibandronate) in PBS. The drug release capacity was highly dependent of the kind of TPS added, as well as of its

particle size. The addition of polyesters, such as PLA, PHB and aliphatic polyester, produced a decrease in the mechanical properties (compression and tensile) and a very limited capacity for water absorption of the system.

Degradable chitosan/ β -tricalcium phosphate (β -TCP) microspheres were used as an added constituent to commercial available PMMA bone cement [169]. Their introduction promoted a significant decrease of the curing peak temperature, increased the setting time and reduced the mechanical properties, namely the ultimate compressive strength and the bending strength. The degradation test of these composites showed that the weight loss can be superior to 20% after 100 days of immersion in phosphate buffer saline solution. SEM observations indicated that these composites could degrade gradually and provide rough and porous spaces for cell growth leading to a more stable structural anchorage of the cement with the surrounding tissues. Following a similar strategy, a bioactive bone cement (BBC), composed of chitosan (10 wt.%) and HA from natural bone powder (trabecular bone blocks of porcine spines), at concentrations of 40, 50 and 60 wt.% was developed [170]. Compared with pure PMMA, the water absorption, weight loss, and porosity increased for the BBCs, but the compressive Young's modulus and the ultimate compressive strength decreased. No cytotoxic characteristics were found associated with any of the BBCs and cell proliferation tests demonstrated that BBC with 50 wt.% of HA was preferable to pure PMMA for cell attachment and proliferation. However, the addition of chitosan (concentrations of 1–5%) into gentamicin-loaded Palacos® R bone cement significantly decreased drug release and did not prevent the bacterial colonisation [171]. The mechanical performance of these cements was significantly reduced after 28 days of saline degradation with the compressive and bending strengths not in compliance with the minimum requirements as stipulated by the ISO standard for PMMA bone cements.

Another interesting approach was the development of a novel class of cements, the so called hydrophilic, partially degradable and bioactive cements (HDBC). These were acrylic bone cements based on corn starch/cellulose acetate blends (SCA), a bioactive ceramic filler (HA or bioactive glass) and a hydrophilic monomer (acrylic acid (AA) or then 2-hydroxyethyl methacrylate (HEMA)) [69, 172-174]. Higher solid/liquid ratio shortened the dough time and decreased the peak of temperature. Mechanical properties were in the range of conventional cements and the best results were obtained with a solid/liquid ratio of 55/45 and an HA content of about 20 wt%. The degradation percentage reached a maximum of 12% after 90 days in saline solution. The formulations developed with HA amounts of at least 20% were clearly bioactive [174]. The results for water uptake increased with increasing amount of the hydrophilic monomer, and could be adjusted to values ranging from 20 to 65wt%. It was found that cements containing AA and bioactive glass ($\text{MgO-SiO}_2\text{-3CaO.P}_2\text{O}_5$) did not show a bioactive behaviour, because of the

deleterious effect of this monomer on the calcium phosphate precipitation on the polymeric surfaces [173]. HEMA did not present this inhibitory effect and the addition of 30 wt.% of bioactive glass to this system promoted the formation of a dense apatite layer after 7 days of immersion in simulated body fluid [172]. This novel concept in terms of bone cement could allow bone ingrowth in the cement, and induce a press-fitting effect, improving the interfaces with both the prosthesis and the bone [69].

CONCLUSIONS AND SCOPE OF THE THESIS

Despite the success, globally recognized, of the use of PMMA bone cements in orthopaedic surgery there are some drawbacks that limit their performance such as non-bone-bonding capability, relatively low mechanical strength, release of unreacted monomer and high curing temperatures. A new generation of acrylic bone cements with better properties than those commercially available is strongly desired in order to ensure the long term clinical performance of the cemented arthroplasties.

As previously shown, a lot of work has been carried out during the last 35 years to study acrylic bone cements and numerous changes in the preparation techniques and composition have been attempted to improve the properties of this material. Although several modifications have been proposed as alternatives to the original formulations, none was successfully introduced in the market. In fact most of the novel formulations had to be abandoned due to unexpected problems making it less adequate than the original cement. Therefore, formulations developed 50 years ago consisting of self-curing PMMA are still the main choice for replacement surgeries.

In this context, the work in this thesis was intended to be a contribution towards the development of an improved formulation of bone cement, aiming at solving some of the main drawbacks of the conventional bone cements. Hence we developed a bioactive bone cement, with bone bonding capability, assessed by cellular tests, able to release ibuprofen at therapeutic concentrations sufficient to blunt the inflammatory response associated to the surgical procedure. Moreover, when a biodegradable filler is added to the formulations, it gradually degrades and provides bone cells adhesion and growth on the cement, indicating that it can further allow a stronger in vivo adhesion to the bone and a better stabilization of the implant.

The thesis is divided in 6 chapters, four of which comprise the results obtained from the research experimental work. Two different methods of preparation were used in this study, i.e. polymerization by thermal route (heat cured materials) and polymerization by chemical route (self cured cements).

Chapter 1

This chapter refers to the state of the art on the subject of the thesis, presenting a review of the literature on acrylic bone cements and on the several approaches employed to improve the properties and the performance of these cements.

Chapter 2

It is addressed the method of preparation by the thermal route. Two published papers are presented: First - *"Preparation and study of in vitro bioactivity of PMMA-co-EHA composites filled with a $Ca_3(PO_4)_2$ - SiO_2 - MgO glass"* published in Materials Science & Engineering C- Materials for Biological Applications, 2008; Second - *"Silicate and borate glasses as composite fillers: a bioactivity and biocompatibility study"* published in Journal of Materials Science: Materials in Medicine, 2011. Thus PMMA-co-EHA composites filled with a silicate glass (CSi) and a borate glass (CB) were prepared by free radical polymerization and it is made a discussion on their in vitro behaviour both in acellular (bioactivity) and in cellular media (biocompatibility).

Chapter 3

This chapter describes the method of cement preparation by the chemical route, the procedure which is also used in the following chapters. One paper, *"Properties and osteoblastic cytocompatibility of self-curing acrylic cements modified by glass fillers"* submitted to Journal of Biomaterials Applications, is included in this part. The effect of glass content (30, 40 and 50%) in self cured cements, incorporating the 4,4-bis(dimethylamino)-benzhydrol activator of reduced toxicity, was assessed. Properties such as curing parameters, residual monomer, water uptake, weight loss, bioactivity, mechanical properties (bending and compressive) and osteoblastic cytocompatibility were investigated.

Chapter 4

The study conducted in this chapter explores the possibility of incorporating ibuprofen (anti-inflammatory agent) into the cement, having a material that simultaneously shows controlled drug release and bioactive behaviour. The in vitro liberation profile, release mechanism and the concentration therapeutic of the drug were some of the properties analyzed in this paper entitled *"Influence of ibuprofen addition on the properties of a bioactive bone cement"*, under revision to Biomedical Materials.

Chapter 5

Finally in this chapter we focused the characterization of cements containing biodegradable and bioactive fillers, which provided improvements in both mechanical and biological behaviour. This chapter includes one paper entitled *"Acrylic formulations containing bioactive and biodegradable fillers to be used as bone cements: properties and biocompatibility assessment"* submitted to Biomedical Materials.

Chapter 6

This chapter contains the general conclusions regarding the overall work carried out under the scope of this thesis, as well as some final remarks and future directions.

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CHAPTER 2

SECTION I:

PREPARATION AND STUDY OF *IN VITRO* BIOACTIVITY OF PMMA-co-EHA COMPOSITES FILLED WITH A $\text{Ca}_3(\text{PO}_4)_2$ - SiO_2 -MgO GLASS

ABSTRACT

The nature of the orthopaedic implant surface affects the interaction with cells and subsequent bone formation. The bone/cement interface in cement-held prostheses is considered to be the main cause of fracture leading to implant revision. It is thought that the introduction of a bioactive phase, such as bioglass, in the cement may permit a more stable interface by encouraging direct bone apposition rather than encapsulation of the implant by fibrous tissue. In this work new poly(methylmethacrylate) (PMMA) based composites filled with 0, 30, 40 and 50 (wt%) of a $\text{Ca}_3(\text{PO}_4)_2$ - SiO_2 -MgO glass, were processed. The prepared composites consist of a poly(methylmethacrylate)-co-(ethylhexylacrylate) (PMMA-co-EHA) matrix filled with a glass (G7), with nominal composition 33.26 CaO, 28.07 P_2O_5 , 23.03 SiO_2 , 15.64 MgO (wt %). The *in vitro* bioactivity of the composites was assessed by determining the changes in surface morphology and composition, by X-ray diffraction (XRD) and scanning electron microscopy coupled with X-ray energy dispersive spectroscopy (SEM-EDS), after soaking in a simulated body fluid (SBF) for periods of up to 21 days at 37 °C. Inductively coupled plasma (ICP) was used to assess the evolution of ionic concentrations in the SBF solution. The results obtained confirmed the growth of a hydroxyapatite (HA) layer on the surface of the prepared composites. As expected, HA formation was faster for composites prepared with higher glass content.

This section is based on the following publication:

Lopes PP, Ferreira BJML, Almeida NAF, Fredel MC, Fernandes MHV, Correia RN. Preparation and study of *in vitro* bioactivity of PMMA-co-EHA composites filled with a $\text{Ca}_3(\text{PO}_4)_2$ - SiO_2 -MgO glass. Materials Science Engineering C. 2008;28:572-577.

INTRODUCTION

Fixation of the majority of prostheses in the past has been performed using poly(methylmethacrylate) (PMMA) bone cement. However, an unresolved problem with using PMMA as bone cement is a thickening of the intervening fibrous tissue layer, which leads to aseptic loosening of the cement in some cases [1, 2]. To improve fixation of PMMA to the host bone, various composites with bioactive materials have been developed and studied [3-5].

Bioactive materials (e.g. glasses, sintered HA, glass-ceramics) are able to bond to living bone through a hydroxyapatite (HA) layer formed onto their surfaces [6, 7]. A similar layer is reported to form on the surface of these materials *in vitro*, after soaking in liquids with ionic concentration similar to the human blood plasma [5, 8-10]. Previous studies performed in glasses from the $\text{Ca}_3(\text{PO}_4)_2\text{-SiO}_2\text{-MgO}$ system, have already shown bulk superficial reactivity in the form of a HA like layer [11-13], when immersed in a simulated body fluid (SBF).

The objective of the present work was to investigate if glasses of the same system could induce bioactivity to a new PMMA based polymeric matrix.

EXPERIMENTAL

Glass preparation

A glass (G7), with nominal composition 33.26CaO, 28.07P₂O₅, 23.03SiO₂, 15.64MgO (in this paper, all the compositions are referred to wt %, unless otherwise stated) was prepared from reagent-grade Ca(H₂PO₄).H₂O, CaCO₃, SiO₂ and MgO. The raw materials were mixed in ethanol for 45 minutes and dried at 70 °C. Batches of 80 g were melted in a platinum crucible at 1550 °C for 1 h in air. The melt was poured onto water and the resultant glass frit was powdered in a planetary mill for 8 h. The milled glass powder had a particle size of approximately 10 µm.

Preparation of the composites

Methyl methacrylate (MMA) and 2-ethyl hexylacrylate (EHA) were obtained from Aldrich Chemical Company. Benzoyl peroxide (BPO) was obtained from Merck. Only the MMA was purified, for extraction of hydroquinone, all the other reagents were used as received. PMMA-co-EHA/G7 composites were prepared by addition of the monomers to 0, 30, 40 and 50 % of the glass, as shown in Table 1. BPO was added to the monomer mixture in a ratio of 2.56 %, as a polymerization initiator.

Table 1: Chemical composition of the PMMA-based composites investigated (wt %).

Sample identification	MMA	EHA	Glass, G7
Composite C5	25	25	50
Composite C4	30	30	40
Composite C3	35	35	30
Matrix M	50	50	-

In vitro assay in SBF

Pieces of 5 x 5 x 3 mm were surface ground, mounted vertically and soaked in 15 mL of *tris*-buffered SBF in sterile polyethylene containers maintained at 37 °C. The SBF solution had a similar composition to that of human plasma, as shown in Table 2, and was previously filtered through a Millipore 0.22 µm system. Soaking periods were 1, 3, 7, 14 and 21 days. The concentrations of calcium (Ca), phosphorous (P), silicon (Si), and magnesium (Mg) were determined for each period by inductively coupled plasma spectroscopy (ICP, Jobin Yvon, JY 70 plus). Formation of the calcium phosphate surface layer was followed by X-ray diffraction (XRD, Rigaku Geigerflex Dmax-C with CuK α radiation) and scanning electron microscopy coupled with X-ray energy dispersive spectroscopy (SEM-EDS, Hitachi S-4100, 25 kV acceleration voltage, beam current 10 µA).

Table 2: Ion concentrations and pH of SBF and those of human blood plasma.

	Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	HCO ₃ ⁻	HPO ₄ ²⁻	SO ₄ ²⁻	pH
Plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5	7.2-7.4
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5	7.25

RESULTS AND DISCUSSION***Changes in SBF composition***

Changes in the concentrations of Ca, P, Si and Mg ions in SBF due to immersion of the composites are shown in Figure 1. Along the first day there is a rapid increase in Ca, P, Si and Mg concentration, for all the composites studied, due to filler dissolution. Between 1 and 3 days a concomitant deposition of calcium phosphate occurs as shown by the fall in the Ca and especially

in P concentrations in composite C5, Figure 1a and 1b. During this period the SBF solution in contact with composites C4 and C3 presented less variation in Ca and P concentrations while Si and Mg concentrations continued to rise. From 3 to 14 days the Ca and P concentrations still decreased, for composite C5. Composites C4 and C3 also presented a minor decrease in the Ca and P profile during this period. During the same period the Si and Mg concentrations continued to increase. Between 14 and 21 days the Ca and P concentration profiles are more stable, with comparatively minor changes for the three composites studied. This fact suggests a reformulation of the calcium phosphate deposits - perhaps with morphological implications - rather than apposition. A later increase in soluble Ca and P, for composite C5, is thought to result from detachment of portions of the deposit.

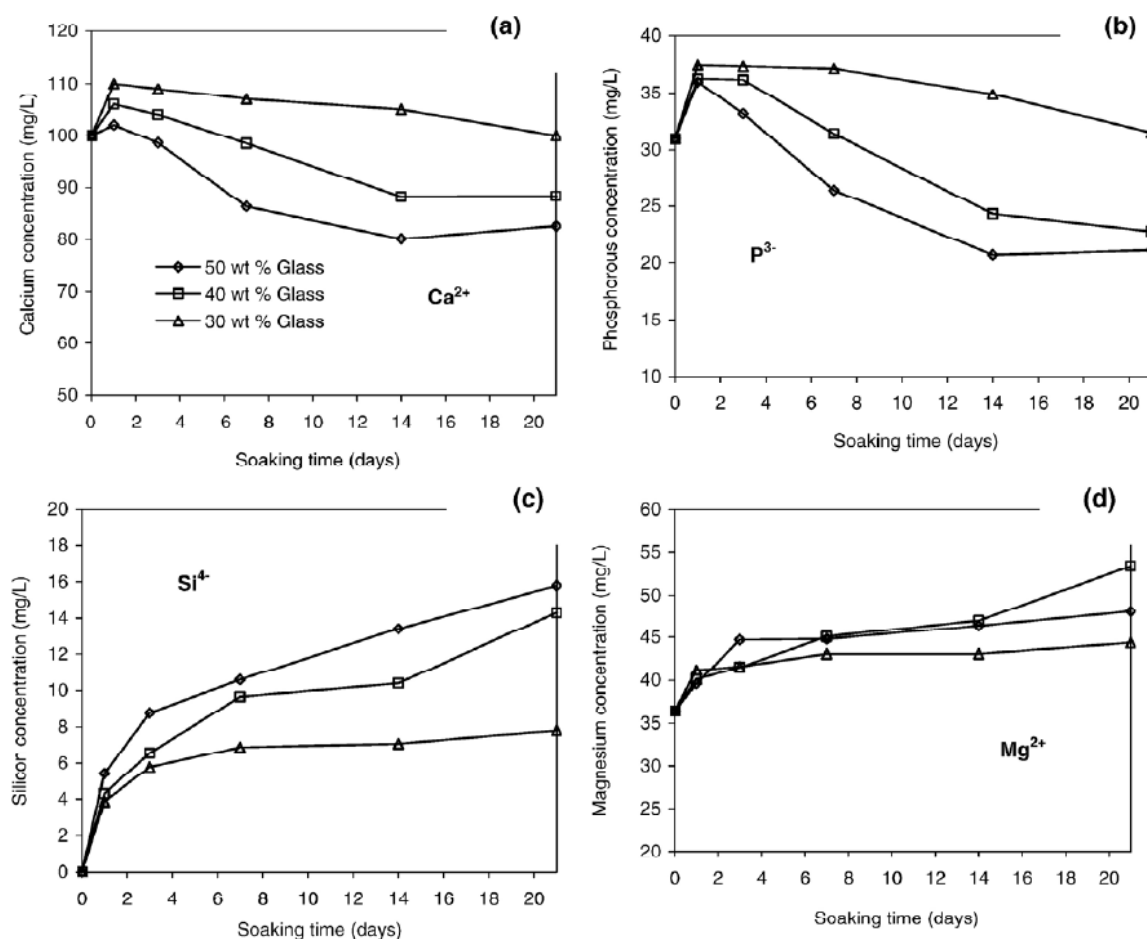


Figure 1: Variation of ionic concentrations of (a) calcium, (b) phosphorous (c) silicon, and (d) magnesium in SBF, during the incubation period.

In general, the ICP analyses reveal that composites prepared with more glass content exhibit a smaller initial increase in Ca and P solution concentrations, Figures 1a and 1b. Usually, one would

expect that composites with higher filler concentration should release more Ca and P into the SBF solution. However, considering the Ca and P concentration profiles we are led to assume that although the composite C5 releases more ions to the solution than C4, as confirmed by the Si analysis shown in Figure 1c, it also induces a more rapid formation of the calcium phosphate layer - resulting in a lower concentration of Ca and P ions in solution. The same can be said about C4 relatively to C3. In a previous study, glasses from the same system in bulk form, showed ability to quickly (less than 1 day) form a calcium phosphate layer, without decreasing the Ca and P solution concentrations in the same length of time [13]. In Mg concentration profiles there are small differences between the composites, the more interesting one comes from comparing C5 and C4 between 14 and 21 days of immersion, since it could result from incorporation of Mg ions, from the SBF solution, into the layer formed on the surface of composite C5.

Formation and characterization of the surface layer

XRD of composite surfaces C5 and C3 during immersion in SBF, are shown in Figure 2. After 1 day in SBF the XRD pattern is still very similar to the pattern before immersion. After 3 days composite C5 exhibits a broad band at $24^{\circ} \leq 2\theta \leq 34^{\circ}$ that suggests a calcium phosphate deposit [14]. This band was also detected for composite C4 (XRD not shown). After the same period the XRD pattern of composite C3 does not evidentiate any new feature. After 7 days in SBF composites C5 and C4 present two diffraction peaks characteristics of synthetic HA [15], for $2\theta = 26$ and 32° , attributed to reflections (0 0 2) and (2 1 1), respectively. Composite C3 still presented, for 7 days, a XRD pattern similar to the one obtained before immersion.

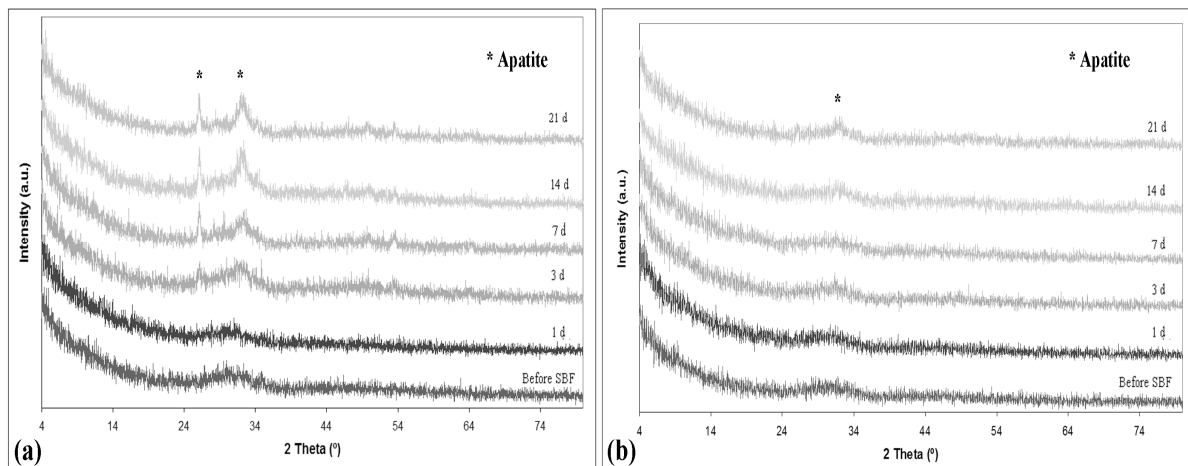


Figure 2: XRD patterns of the surface of composite (a) prepared with 50% and (b) 30% (wt) of glass, during the immersion period.

Between 14 and 21 days of immersion composites C5 and C4 present increased intensity of reflections previously detected while other HA peaks for $2\theta = 50^\circ$ and 53° - attributed to reflections (2 1 3) and (0 0 4), respectively - become evident. For composite C3 the appearance of the broad band characteristic of calcium phosphate deposit was only detected for 21 days of immersion.

SEM images, Figure 3, revealed an almost complete coverage of the surface of composites C5 and C4 after 3 days in the SBF solution. After 7 days a layer of spherical particle aggregates fully covers C5 and C4 surfaces.

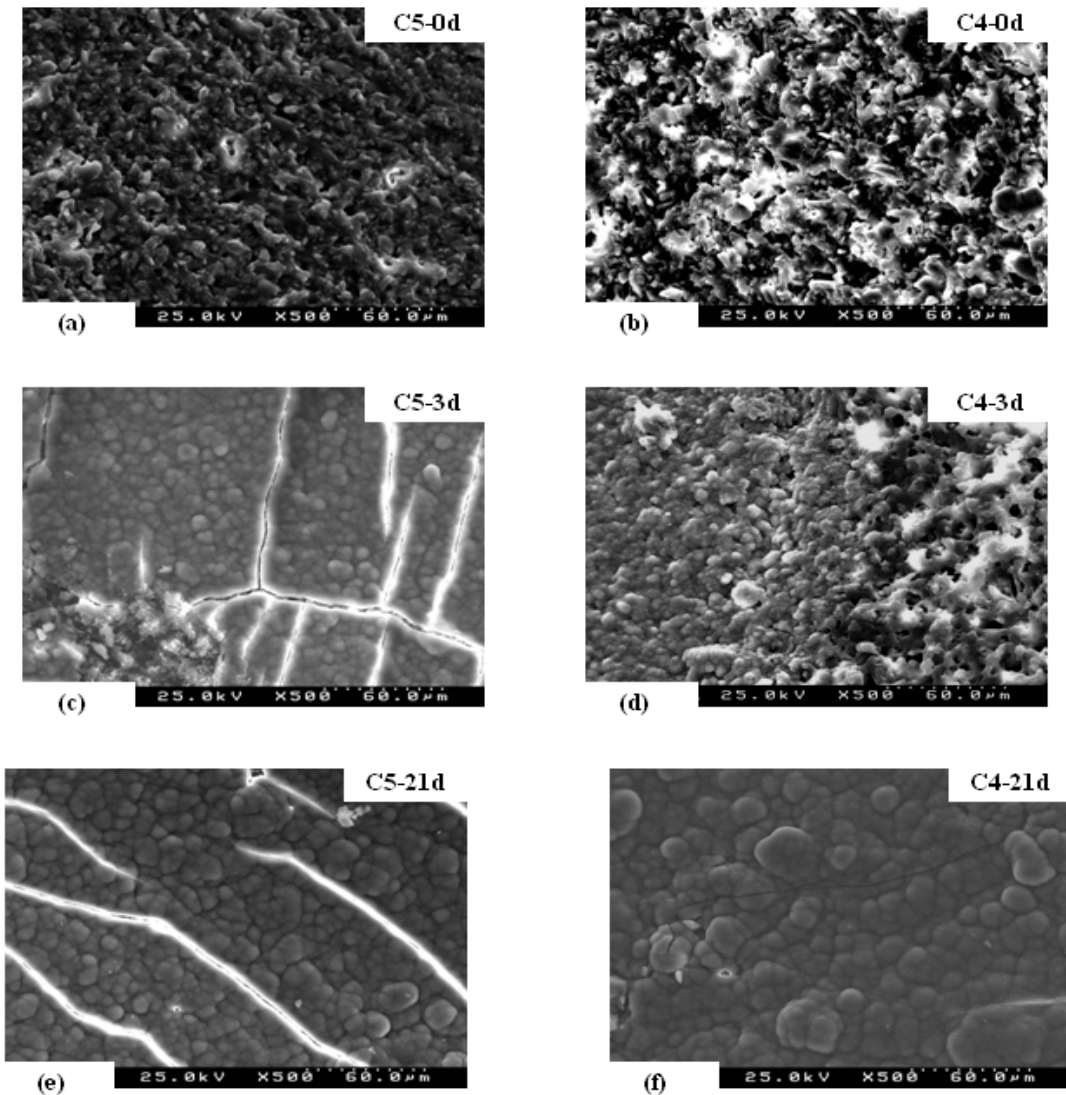


Figure 3: SEM micrographs of the surface of composites C5 and C4 before and after soaking in SBF for 3 and 21 days.

These aggregates consist of numerous acicular crystallites, Figure 4. The morphology of the deposits suggests the formation of a HA-like layer [14, 16]. Between 14 and 21 days in SBF the

HA surface layer seems to exhibit morphological rearrangements - the spherical particles found at 21 days presented a less acicular morphology than those formed after 14 days of immersion, as shown in more detail in Figure 4. It is known that the presence of Mg^{2+} ion in the apatite network decreases its crystallinity [17].

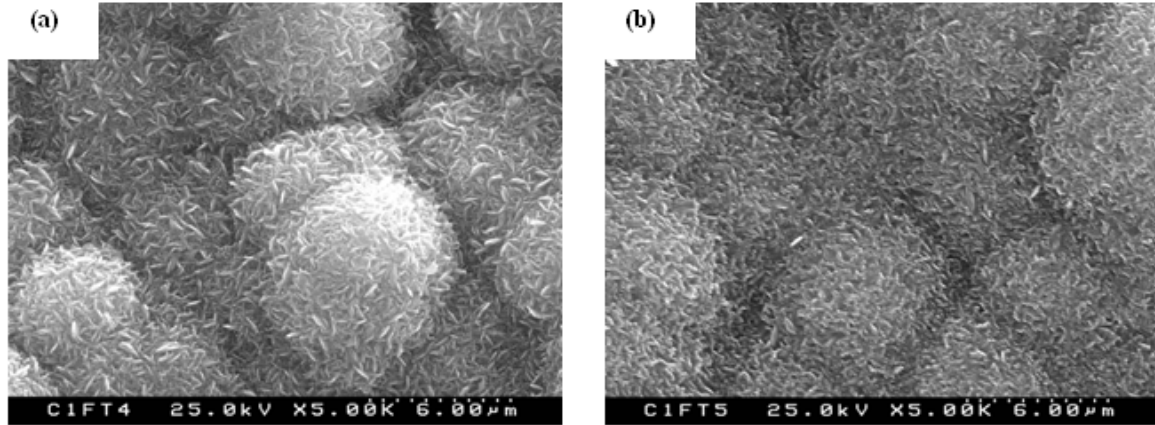


Figure 4: Detail SEM micrographs of the HA layer formed on the surface of composite C5 after soaking in SBF for (a) 14 and (b) 21 days.

The EDS pattern for composite C5, Figure 5, reveals that after 1 day immersed in SBF some of the filler material has dissolved – the Ca, P and Mg signal decreased during this period. The Si signal also decreased, between 0 and 1 day of immersion, but in much less extension. From 1 to 3 days the composite surfaces exhibit a pronounced increase in the Ca and P signal and a corresponding attenuation of the Si and Mg signals, evidencing calcium phosphate deposition. The deposition rate increases between 3 and 7 days since the analysis of the surface of C5 for 7 days only detected the presence of Ca and P. For 7 days of incubation the Ca/P molar ratio obtained by EDS, was 1.69. This value is very similar to the Ca/P molar ratio characteristic of stoichiometric HA - 1.67 [16]. After 14 days in SBF the presence of Si and Mg, besides Ca and P, was again detected. This reversal may be attributing to either the detachment of portions of the calcium phosphate layer or the incorporation of these ions into the surface layer. The presence of sodium was also detected for this soaking time.

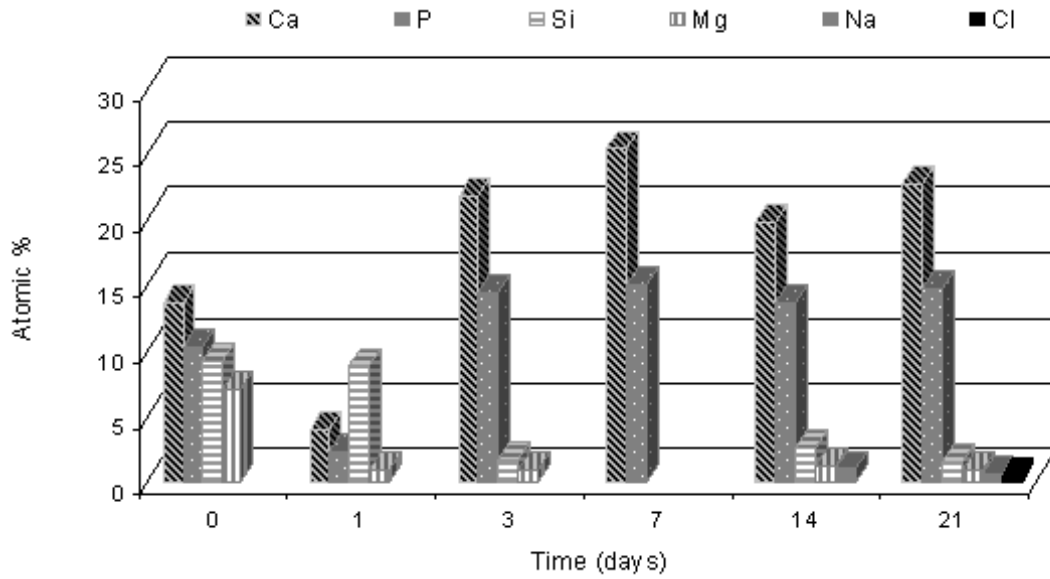


Figure 5: EDS pattern of the surface of composite C5 during immersion period.

The Ca/P molar ratio for the deposit on composite C5 after 14 days of immersion (1.44) is lower than that obtained for 7 days; however, if we assume that the alien ions detected could be incorporated into the HA network, we obtain a new cation/P molar ratio of 1.58, as shown in Table 3. Previous studies confirmed the possibility of these substitutions during the immersion of HA and other related calcium phosphates in SBF [14, 18]. After 21 days of immersion chloride was also found at the surface of composite C5. There are reports of the partial incorporation of this ion into apatite prepared from water solutions with higher chloride concentration [17].

Table 3: Ca/P molar ratios, obtained by EDS, during the immersion of composite C5.

Immersion time (days)	Ca/P	(Ca+Na+Mg)/P
3	1.52	1.58
7	1.69	1.69
14	1.44	1.58
21	1.53	1.62

CONCLUSIONS

The investigated composites, based on a new PMMA-co-EHA (50/50) matrix filled with 0, 30, 40 and 50% (wt) of G7 glass - 33.26% CaO, 28.07% P₂O₅, 23.03% SiO₂, 15.64% MgO (wt.%), revealed their capability for the precipitation of a calcium phosphate layer after soaking in SBF. Structural and morphological characterization of the surface layer, by XRD combined with SEM, indicated that it consists of an HA-like deposit. As expected, the formation of the HA surface layer was faster for composites with higher glass content – 50% and 40%. Composite surfaces were completely covered after 7 days in SBF. Changes in SBF composition combined with EDS analysis suggest the incorporation of Mg²⁺ ions in the surface layer after 14 days of immersion. The results obtained are encouraging and suggest that this new bioactive composites could be an alternative to the typical PMMA bone cements used for fixation of orthopaedic implants.

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CHAPTER 2

SECTION II

SILICATE AND BORATE GLASSES AS COMPOSITE FILLERS: A BIOACTIVITY AND BIOCOMPATIBILITY STUDY

ABSTRACT

Composites filled with a silicate glass (CSi) and a new borate glass (CB) were developed and compared in terms of their in vitro behaviour both in acellular and cellular media. Acellular tests were carried out in SBF and the composites were characterized by SEM-EDS, XRD and ICP. Biocompatibility studies were investigated by in vitro cell culture with MG-63 osteoblast-like and human bone marrow cells. The growth of spherical calcium phosphate aggregates was observed in acellular medium on all composite surfaces indicating that these materials became potentially bioactive. The biological assessment resulted in a dissimilar behaviour of the composites. The CSi demonstrated an inductive effect on the proliferation of cells. The cells showed a normal morphology and high growth rate when compared to standard culture plates. Contrarily, inhibition of cell proliferation occurred in the CB probably due to its high degradation rate, leading to high B and Mg ionic concentration in the cell culture medium.

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INTRODUCTION

Acrylic polymers have been extensively used in orthopaedic and dental applications as filling and fixing agents [1]. However, a long-term problem associated with this material is the formation of fibrous tissue at the bone-cement interface which may compromise the fixation of the prosthesis [2]. Thus, over the years, a lot of research effort has been put into optimization of the properties of these materials [1,3,4].

The main condition for a synthetic material to form a stable bond with the bone is the precipitation of an apatite layer on its surface, which is responsible for its bioactivity [5]. Several compositions of glasses may promote this behaviour and they can be used to improve the bone bonding capability of inert matrices like PMMA.

According to previous work, the formation of hydroxyapatite (HA) seems to be induced by functional groups existing on material surface, such as Si-OH [6]. So, it is accepted and well characterized that glasses like 45S5 (Bioglass®) form a silica-rich gel layer (Si-OH), through ion exchange reactions with the physiological medium followed by precipitation of calcium and phosphate ions, resulting in a bioactive material, which enhances bonding capability with bone and soft tissues [7-9].

Recent researches demonstrated that borate-based glasses derived from the 45S5 glass by fully or partially replacing the SiO₂ with B₂O₃, can be converted into hydroxyapatite when placed in dilute phosphate solution, being the conversion rate to HA more rapid for the glasses with higher B₂O₃ content [10,11]. It is believed that the formation of apatite on B-based glasses follows a set of dissolution-precipitation reactions similar to those of a Si-based glass, but the silica gel layer is absent [10].

The biological performance of the B-based glasses has been addressed in previous studies. In vivo, particles of a boron-modified 45S5 glass containing 2 wt% of boron oxide, implanted in rat tibia bone marrow, promoted new bone formation and a significant increase of the thickness of osseointegrated tissue when compared with control 45S5 glass [12]. Also, in vitro, boron-modified 45S5 glass containing varying amounts of B₂O₃, tested as-prepared and partially converted to HA (by soaking in a K₂HPO₄), allowed the proliferation of osteoblast cells, although inhibition of cell growth was observed for the glasses with higher B₂O₃ content, especially for the as-prepared samples and tested in static conditions [13]. In this context, it seems useful to investigate new materials and expand the range of glass compositions available for use in biological applications and the B-based glasses might be a promising material for biological applications.

In terms of filler, innovative studies published by the present group showed that some glasses of the 3CaO.P₂O₅-MgO-SiO₂ system have the potential to be used as biomaterial [14] and the high

MgO content in the composition of these glasses does not hinder their apatite forming ability [15,16]. Therefore further investigation into its biological behaviour will be of great interest, since the effect of this filler on the proliferation of cells has not been disclosed.

In this context, the main purpose of the present work is to analyse the behaviour of PMMA-co-EHA composites filled with silicate (CSi) and new borate (CB) glasses, in acellular and in cellular media, regarding their capability for calcium phosphate formation and osteoblast cell proliferation and differentiation. The proposed borate glass composition was prepared by replacing all the silicate of the silica based glass by borate, thus becoming to our knowledge the only B-based glass with addition of MgO, for biomedical application, found in the literature. Bioactivity of the composites was assessed in SBF and cytocompatibility studies were performed firstly with the osteoblast-like MG63 cell line for a rapid screening assay and, afterwards, with human bone marrow cells to assess the performance of the material regarding osteoblastic proliferation and differentiation events.

MATERIALS AND METHODS

Preparation of the glasses

Two different glass compositions were used in the experiments. A silicate glass composed of (mol%) 38% CaO, 12.7% P₂O₅, 24.8% MgO, 24.5% SiO₂ and a borate glass, which consists of a similar composition where SiO₂ was entirely replaced by B₂O₃. The glasses were prepared through the classic melt-quenching method and the resultant glass frit was dry-milled (Retsch, RM100 Mortar Grinder Mill) to a powder with mean particle size of 10 µm, measured with a Coulter LS Particle Size Analyzer. The amorphous character of the glasses was confirmed by X-ray diffraction (XRD, Rigaku Geigerflex Dmax-C with CuKα radiation).

Preparation of the composites

PMMA/EHA/Glass composites in a ratio (wt%) of 25:25:50 respectively, were synthesized and compared according to the glass composition [16]. Methyl methacrylate (MMA) and 2-ethyl hexylacrylate (EHA) were obtained from Aldrich Chemical Company and Merck supplied benzoyl peroxide (BPO). All the reagents were used as received. The composites were prepared by free radical polymerization at 80 °C for 24 h and no activating agent was used. The monomers were first mixed in a glass recipient, afterwards BPO (polymerization initiator) was dissolved in this liquid mixture and finally the glass was incorporated into the mix. It was poured into a Teflon mould where polymerization took place. Samples of 5×5×3 mm³ were prepared for the bioactivity and biocompatibility studies, and sterilized by 70% alcohol.

In vitro bioactivity

In order to evaluate the in vitro bioactivity and compare the degree of apatite formation on the composites, specimens were mounted vertically and soaked in simulated body fluid (SBF) at physiological conditions of temperature and pH, respectively, 37 °C and 7.4. The SBF solution was prepared according to the formulation of Kokubo and Takadama [17], with ion concentrations nearly equal to those of human blood plasma (Table 1). This solution was previously filtered through a Milipore 0.22 µm system and it was used a constant specimen surface area to solution volume ratio of 0.1 cm⁻¹. The materials were soaked for periods of 1, 3, 7, 14 and 21 days. After immersion the samples were removed from the fluid and their crystallinity, morphology and surface modification were followed by X-ray diffraction (XRD) and scanning electron microscopy coupled with X-ray energy dispersive spectroscopy (SEM-EDS, Hitachi S-4100, Japan) at an acceleration voltage of 25 keV and beam current of 10 µA. The solution was characterized by inductively coupled plasma spectroscopy (ICP, Jobin Yvon, JY70 Plus) to measure ionic concentration and pH was evaluated at the different times.

Table 1: Ionic Concentrations (mM) of SBF and Human Blood Plasma.

	Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	HCO ₃ ⁻	HPO ₄ ²⁻	SO ₄ ²⁻
Plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5

Biocompatibility studies

MG63 osteoblast-like cells

The MG63 cell line, originally derived from a human osteosarcoma, has shown numerous osteoblastic features, being largely used in biocompatibility tests [18]. These cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air, in α-Minimal Essential Medium (α -MEM) containing 10% fetal bovine serum, 50 µg/mL ascorbic acid, 50 µg/mL gentamicin and 2.5 µg/mL fungizone. For subculture, PBS (phosphate-buffered saline) was used to wash the cell monolayer twice, which was then incubated with trypsin – EDTA solution (0.05% trypsin, 0.25% EDTA) for 5 min at 37 °C to detach the cells. Cells were resuspended in culture medium and cultured (2x10⁴ cell cm⁻²) for 7 days in standard polystyrene culture plates (control) and on the surface of the composites. The medium was changed every 2–3 days. Control cultures and seeded material

samples were evaluated at days 1, 3, and 7 for cell viability/proliferation and observed by confocal laser scanning microscopy (CLSM; Leica SP2 AOBS).

Human bone marrow cells

Human bone marrow, obtained from orthopaedic surgery procedures (after patient informed consent), was cultured in the same experimental conditions as those used in the culture of MG63 cells. Primary cultures were maintained until near confluence (10–15 days) and, at this stage, adherent cells were enzymatically released (trypsin–EDTA solution). The cells were seeded at a density of 2×10^4 cell/cm² in control conditions (standard plastic culture plates) and on the surface of the composites. Control cultures and seeded material samples were cultured for 21 days in the presence of 50 µg/ml ascorbic acid, 10mM β-glycerophosphate and 10 nM dexamethasone, experimental conditions reported to allow the osteoblast differentiation in this culture system [19]. All the experiments were performed in the first subculture, since the sequential passage of bone marrow cells results in a progressive loss of the osteoblastic phenotype [20]. Control cultures and colonized composites were evaluated throughout the culture time for cell morphology, cell viability/proliferation, alkaline phosphatase (ALP) activity and ability to form calcium phosphate deposits, as follows.

Cell viability/proliferation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a simple colorimetric method to measure cytotoxicity and viability/proliferation, first developed by Mosmann [21]. This method is based on the capacity of viable cells to metabolize tetrazolium salt by forming purple formazan crystals, which can be dissolved and quantified by measuring the absorbance of the solution at 600 nm. Cultures were incubated with 0.5 mg/mL of MTT in the last 4 h of the tested culture period; the medium was then decanted, formazan salts were dissolved with 200 µL of dimethylsulphoxide and the absorbance was measured in an ELISA reader. Results were compared in terms of macroscopic surface area and expressed as Acm^2 .

Alkaline phosphatase activity

Alkaline phosphatase is a glycoprotein that participates in processes leading to mineral formation in tissues like bone [22]. ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton in water) and assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (p-nitrophenol) at $\lambda=405$ nm: Hydrolysis was carried out for 30 min at 37 °C. Results are expressed in nanomoles of p-nitrophenol produced per min per µg of protein ($\text{nmol} \cdot \text{min}^{-1} / \mu\text{g protein}$).

SEM and CLSM microscopy

The samples for SEM observation were fixed in 1.5% glutaraldehyde in 0.14M sodium cacodylate buffer (pH 7.3), then dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analysed in a JEOL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDS) microanalysis capability (voyager XRMA System, Noran Instruments).

For CLSM assessment, the samples were fixed in 3.7% paraformaldehyde (10 min). Cell cytoskeleton filamentous actin (F-actin) was visualized by treating the cells with Alexa Fluor90 488 Phalloidin (1:20 dilution in PBS, 1 h) and counterstaining with propidium iodide (1 $\mu\text{g.mL}^{-1}$, 10 minutes) for cell nuclei labelling. Labelled cultures were mounted in Vectashield® and examined with a Leica SP2 AOBS (Leica Microsystems) microscope.

Statistical analysis

Values are expressed as mean \pm standard deviation (SD) of three replicates and were compared using the student's *t*-test, with a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

In vitro Bioactivity

Bioactivity of CSi and CB composites was assessed in SBF, which is widely employed as synthetic plasma, unlike most of the reported studies regarding the bioactivity of the B-based glasses [10,11,13,23], that used a 0.02M K_2HPO_4 solution (with a phosphate ions concentration approximately 20 times that of the human blood plasma).

SEM images of CSi and CB composites after immersion in SBF for various periods are shown in Figure 1. Similar results were obtained for the two composites, prepared with different glass compositions, clearly showing the precipitation of a surface layer on both.

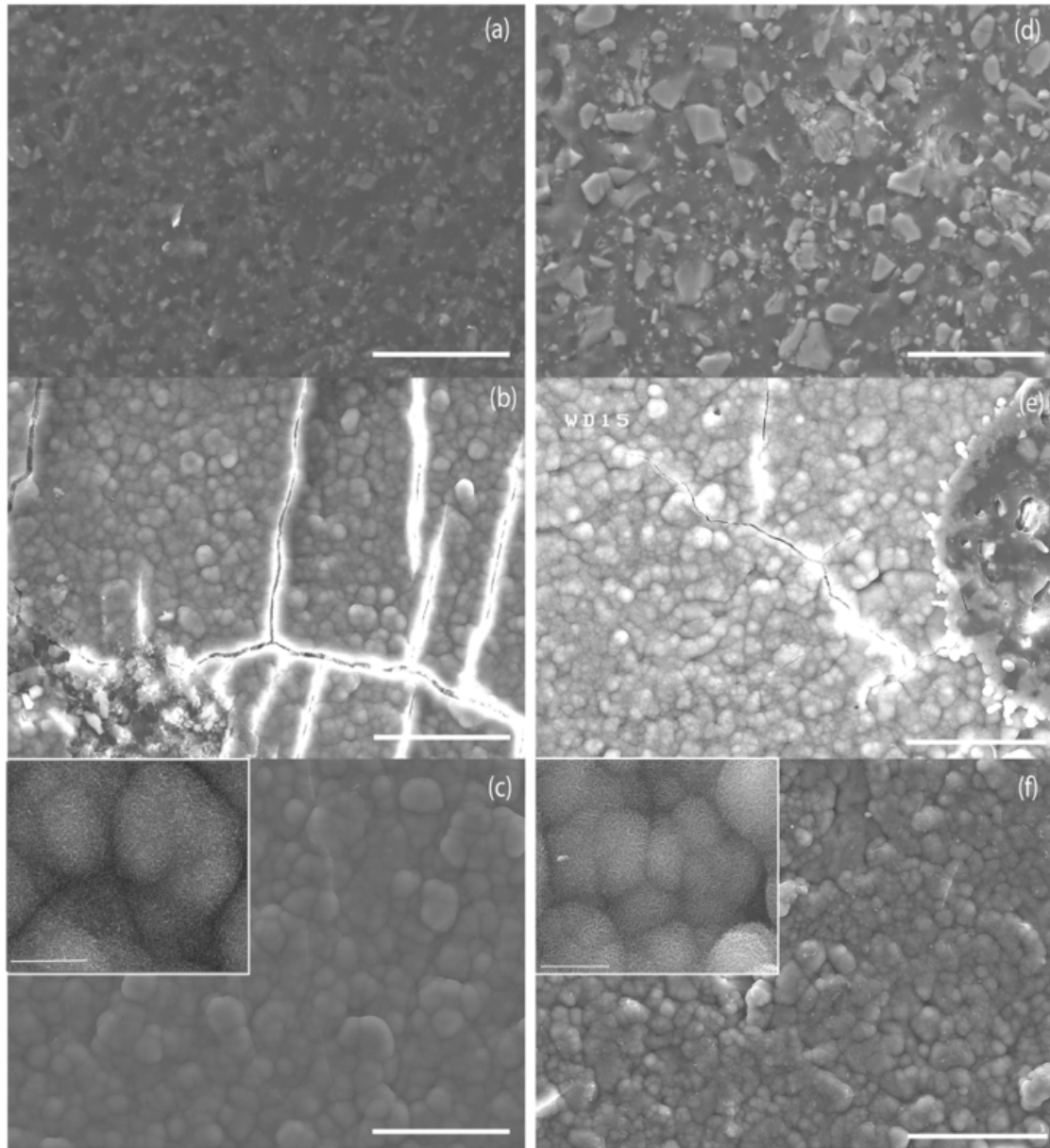


Figure 1: SEM micrographs, for the CSi composite (a) before immersion, (b) after 3 days and (c) after 7 days in SBF. For CB, (d) before immersion, (e) after 3 days and (f) after 7 days. Bars 60 μm (insert: bar = 6 μm).

As seen in Figure 1 the initial samples (before immersion) were constituted by the glass particles dispersed in the polymeric matrix. This morphology changed with the soaking time and, after 3 days in SBF, the spherical precipitates began to grow and partially cover the surface of the composites. After 7 days, the composites were covered with a homogeneous layer of precipitates which consisted of numerous needle-like crystalline aggregates characteristic of hydroxyapatite [24]. With further increase in the soaking time, there was no significant change in the surface morphology, and a layer identified as calcium phosphate could still be observed after 14 and 21 days (not shown).

The surface chemical analysis was carried out by EDS and the patterns of the composite surface composition before and after soaking in SBF are depicted in Figure 2. The obtained results for 0 days confirmed that the elements that constitute the composites before immersion are in agreement with the glass composition. The CB composite revealed the presence of Ca, P and Mg (B is not detectable by EDS) and for CSi, besides these same elements, Si was also identified. The surface of composites CB and CSi exhibited an increase in Ca and P concentration and a corresponding decrease of the Mg and Si signal (when it is present) after 3 days of immersion, evidencing a calcium phosphate formation. Furthermore, for the CB composite the presence of a small amount of Cl ions incorporated in the mineral phases was detected, probably coming from the SBF. The surface analysis of the composite CSi, for 7 days, only showed the occurrence of Ca and P, and the Si and Mg signal disappeared. For CB, after 7 days, the spectrum was similar to earlier time. The measured Ca/P molar ratio for this immersion time was 1.69 for CSi and 1.57 for CB.

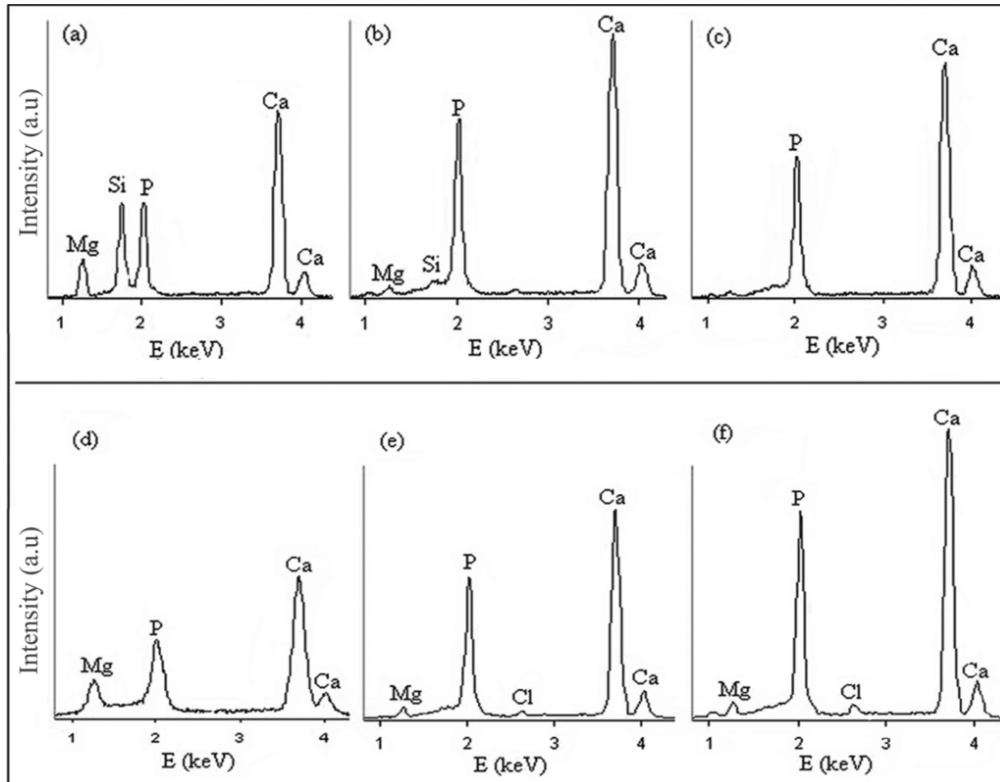


Figure 2: EDS results, for CSi (a) before immersion, (b) after 3 days and (c) after 7 days. For CB (d) before immersion, (e) after 3 days and (f) after 7 days.

XRD patterns of composites after the several soaking periods are illustrated in Figure 3. The composites without immersion showed typical spectra of amorphous phase and absence of detectable crystalline phase. Peaks accusing crystallinity of the surface precipitates began to appear after 3 days of immersion and sharpened for the longest period. The results indicate that a calcium

phosphate was deposited on the surface of CSi, being identified the peaks characteristic of hydroxyapatite at $2\theta = 26^\circ, 32^\circ, 50^\circ$ and 53° attributed to reflections (0 0 2), (2 1 1), (2 1 3) and (0 0 4) respectively [25]. The intensity of these peaks increased with soaking time, due to the growth, on the composite surface, of an apatite layer of enhanced crystallinity with time. The CB spectrum revealed the presence of Mg-substituted tricalcium phosphate phase (whitlockite) together with the apatite phase in the newly formed layer. After 3 days, the peaks of diffraction for $2\theta = 28^\circ, 31^\circ$ and 35° were assigned to the reflection (2 1 4), (0 2 10) and (2 2 0) of whitlockite. The apatite phase was detected after 7 days immersion through the peaks at $2\theta = 26^\circ, 32^\circ, 53^\circ$. The precipitation of two phases (apatite and whitlockite) was also identified on the surface of sol-gel glasses containing Mg when exposed to SBF [26,27].

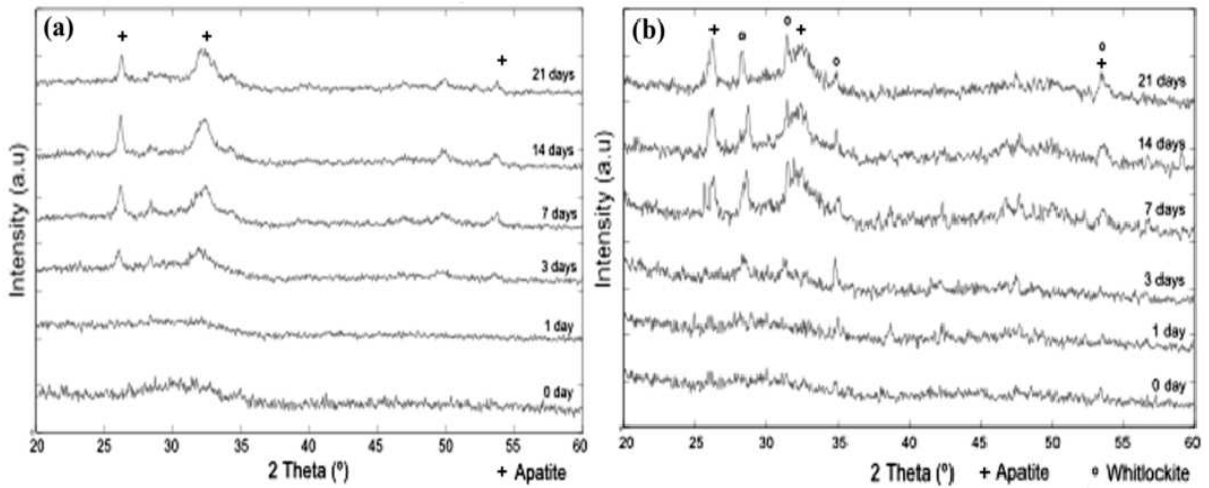


Figure 3: DRX patterns of composite (a) CSi and (b) CB.

When magnesium is incorporated into the atomic structure of HA, the central calcium atom is substituted by magnesium. Since the ionic radius of Mg^{2+} (0.69 \AA) is considerably smaller than that of Ca^{2+} (0.99 \AA), the placement of Mg into Ca position distorts the HA structure, resembling more to whitlockite [28]. Prolonged soaking time of the composite arose the apatite phase herewith whitlockite.

Variation of ions concentration and pH in SBF solution versus soaking time of the composites are presented in Figure 4. For CSi composite, the release of Ca and P occurred during the first period of immersion due to the dissolution of the glass followed by their consumption for the growth of calcium phosphate layer, resulting in the observed decrease in the concentrations of these ions in the solution. The Mg and Si concentration slightly increased as a result of glass dissolution and ionic exchange with the solution. For CB composite, the release of Ca and P showed a similar profile to CSi indicating that these ions are required for the build up of the calcium phosphate layer

on the composite. The Mg and B concentration in solution increased continuously up to 14 days, reaching values much higher than those regarding CSi.

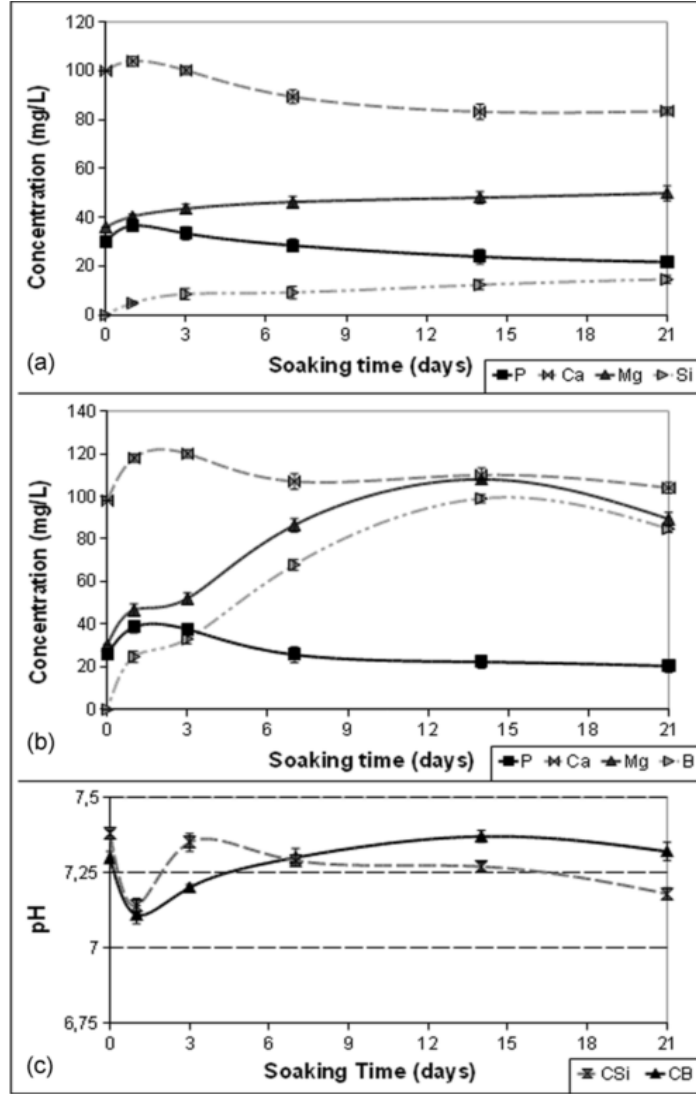


Figure 4: Variation of ionic concentration in SBF due to immersion of (a) CSi and (b) CB, and (c) pH evolution with time.

The less cohesive structure of borate glass compared to the silicate glass can be responsible for its higher degradation rate. Despite the high dissolution of the borate glass, the pH did not change significantly until the end of the test. The elevated Mg concentration in SBF for this composite can explain the formation of both apatite and whitlockite phases detected by XRD and consequently the presence of its signal in EDS for all soaking periods. For other bioactive materials it is reported that when the Mg/Ca molar ratio of the solution is higher than 0.05, a Mg-substituted TCP can be formed [29].

Biological Assessment

As-prepared CSi and CB composites were seeded with MG63 osteoblast-like cells for a preliminary and quick screening. Results are presented in Figure 5. Regarding the MTT assay, control cells, seeded in standard polystyrene culture plates, presented a lag phase followed by an increase in the cell proliferation, especially from days 3 to 7. Comparatively, seeded CSi displayed significantly higher values at day 1, suggesting that a higher number of cells attached to the composite, and, afterwards, cells proliferated with a similar growth rate, resulting in higher MTT reduction values throughout the entire culture time. By contrast, seeded CB composite presented low MTT values at day 1, and, following, cell proliferation increased slowly during the culture time. CLSM observation of the cultures is in line with the MTT assay. At day 7, seeded CSi was completely covered by a continuous and thick well-organized cell layer, whereas CB showed only few and altered attached cells.

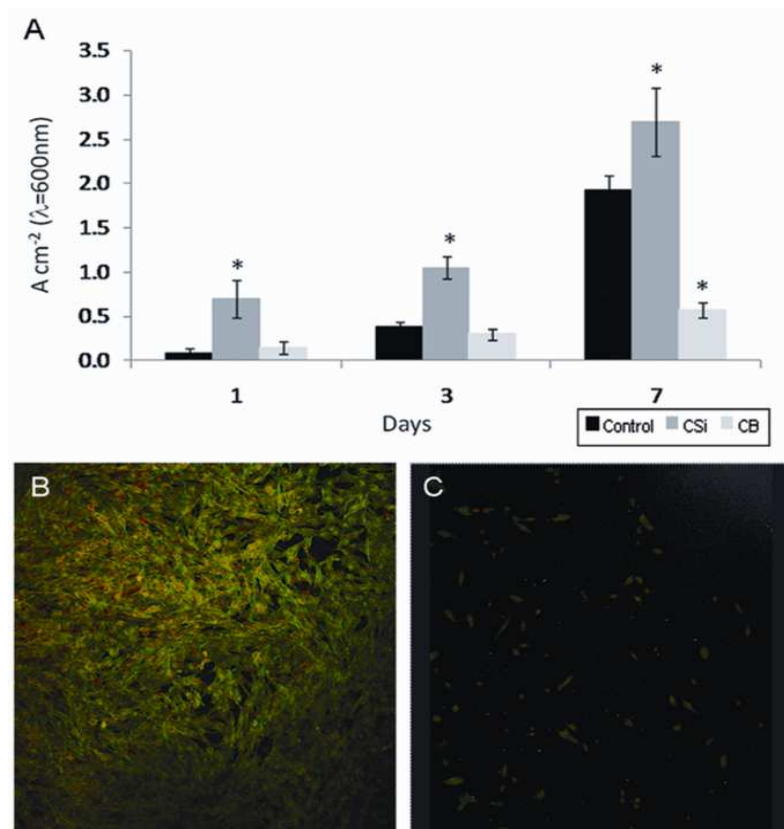


Figure 5: Behaviour of MG63 osteoblast-like cells cultured up to 7 days over CSi and CB composites. (A) Cell viability/proliferation, estimated by MTT assay, (*) Significantly different from control culture. CLSM images at 7 days, on (B) CSi and (C) CB.

In addition, CSi and CB composites were seeded with human bone marrow cells and were cultured in conditions known to favour osteoblast differentiation [19]. Observation of the seeded materials by CLSM (Figure 6) showed that, following cell plating, cells attach to the material surface within minutes. Over CSi, at 1 h, attached cells showed varying degrees of cytoplasm expansion, displaying an elongated morphology at 24 h with a well defined nucleus and F-actin cytoskeleton. Cells proliferated throughout the culture time and, at day 21, the material surface was covered with a continuous and well organized cell layer.

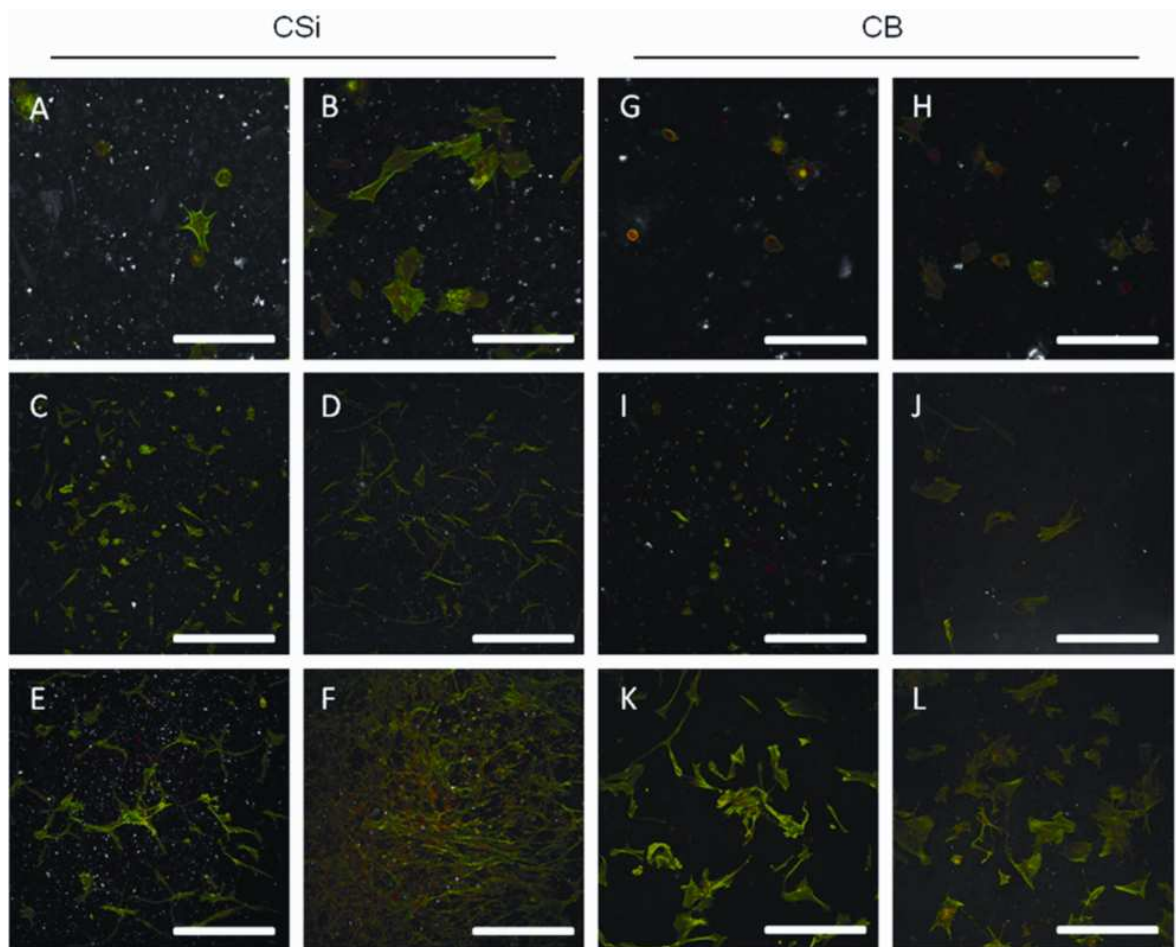


Figure 6: CLSM observation, for CSi composites seeded with human bone marrow cells and cultured for (A) 1 hour, (B,C) 24 hours, (D) 7 days, (E) 14 days and (F) 21 days. For CB composite (G) 1 hour, (H, I) 24 hours, (J) 7 days, (K) 14 days and (L) 21 days. A, B, G and H: bar, 60 µm; C – F and I – L: bar, 500 µm.

The MTT assay, Figure 7 confirmed this behaviour, i.e., an increase in the cell viability/proliferation during the culture time. In addition, cells presented a high ALP activity, which increased significantly during the third week suggesting that the growing cells were engaged with an osteoblast differentiation process [20,30].

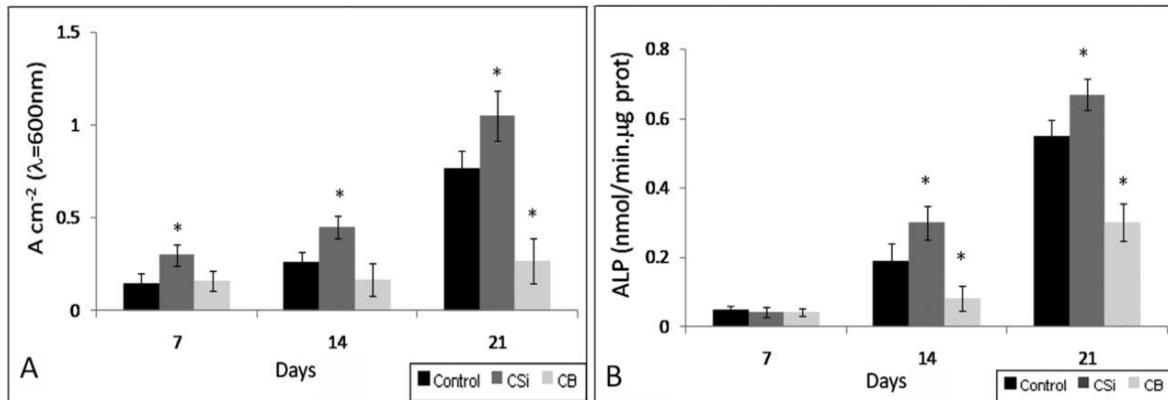


Figure 7: Cell viability/proliferation by MTT assay (A), and alkaline phosphatase activity (B) of human bone marrow cells grown over CSi and CB for 21 days. (*) Significantly different from control culture.

Accordingly, SEM observation of colonized CSi composite at day 21 showed a thick cell layer with a fibrillar matrix and associated calcium phosphate mineral deposits, Figure 8, a proof of the complete expression of the osteoblast phenotype [19]. By contrast, cells cultured over CB composite showed signs of deleterious effects regarding the cell adhesion process, reflected by a low number of attached cells at 1 h and impaired cytoplasm expansion at 24 h. Cell proliferation was also impaired with only small cell clusters scattered over the surface at day 21. ALP activity was also lower than that on CSi, and matrix mineralization did not occur. Results for CB composite are shown in Figures 6-8.

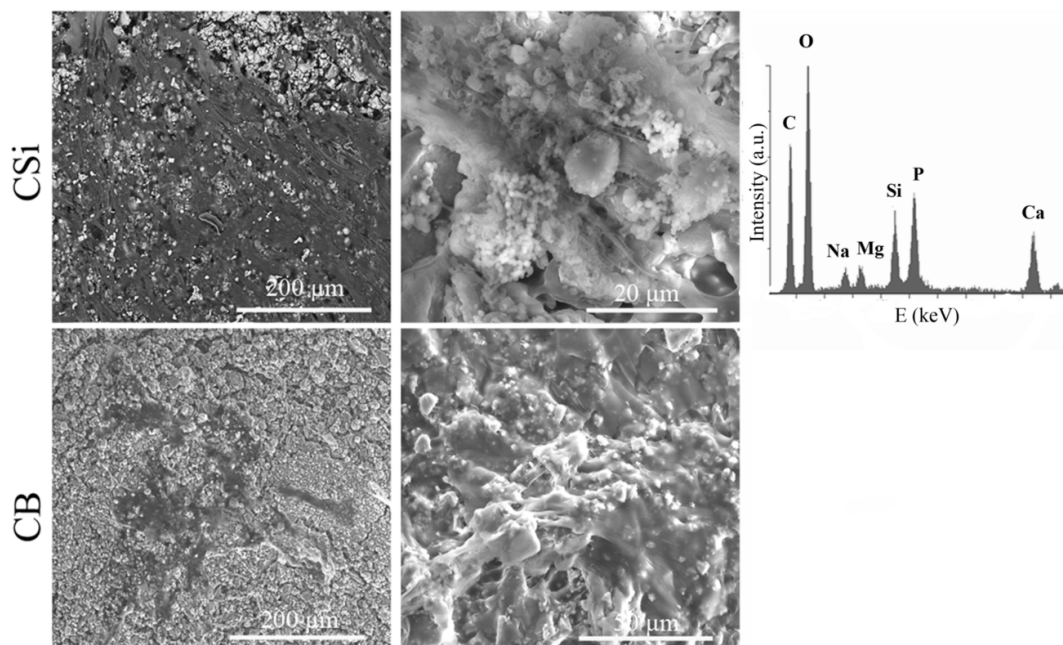


Figure 8: SEM appearance of human bone marrow cells cultured for 21 days over CSi composite, inset: EDS spectrum of the mineralized structures, and CB composite.

As mentioned before, CSi and CB composites were seeded “as-prepared” and the differences regarding the biological performance might be related to the behaviour of the composites following cell culture. Exchange reactions between the material surface and the culture medium such as dissolution/precipitation reactions along with the simultaneous adsorption of biologically active molecules such as peptides and proteins creates a specific microenvironment that can positively or negatively influence cell adhesion and the subsequent proliferation and differentiation events [31-33]. Results reported in the previous section showed a different behaviour of CSi and CB following immersion in SBF, suggesting that differences in the chemical composition and dissolution of the glasses have an important impact on cell growth.

The improved biological performance of CSi compared with CB is related with its lower degradation rate, which is directly correlated to the chemical composition and structure features of the glass used as filler in percentages of 50 %wt. The structure and network connectivity strongly determine the solubility and bioactivity of the glass showing that the adhesion and proliferation of the cells on the composite filled with silicate glass is favoured. In addition, the positive effect of CSi on cell behaviour may also be related to the release of silicon, which plays an important role in physiological process during the growth and development of the bone, acting on the proliferation and differentiation of osteoblasts [34]. The levels of the released Si (20 ppm) are within a wide range, between 0.1 and 100 ppm, which leads to a dose dependent increase of human osteoblast-like cells’ proliferation and differentiation, in short-term cultures [35], and within the variable physiological range in humans [36].

In the CB composite, the structure of the borate glass is less cohesive and therefore more soluble, resulting in two apparently competing effects: the formation of calcium phosphate layer improving biological performance, and the high release of ions into the cell culture medium causing a greater inhibition of cell proliferation. As suggested by the results found in SBF, B and Mg ions in the culture medium released from CB may attain levels high enough to cause cytotoxicity. Previous in vitro works demonstrated that borate glass resulted in a greater inhibition of cell proliferation under static culture conditions, if the boron concentration was above a certain threshold value [13,23]. Although it has already been reported the whitlockite stimulation of cell proliferation and the synthesis and secretion of collagen [37], the ICP measurements showed an abnormal concentration of magnesium, which together with B may result in a negative effect on cell proliferation.

Results provided in vitro evidence of poor biocompatibility for this composite. However, it is believed that, in vivo, the effects of ion release might be less severe than those seen in the cell cultures experiments performed under static conditions. The body fluids represent a more dynamic system in which the local chemical changes are attenuated by metabolic processes and by the

continuous circulation, preventing the excessive level of ions at the interface cell/material. Moreover, in living body, upon the implantation of a material in the bone tissue, there is a continuous availability of osteoblast progenitor cells that can adhere to the material surface when the appropriate sets of conditions are met. These factors most probably will increase the performance of the CB composite.

CONCLUSIONS

The investigated composites, CSi and CB, promoted the growth of spherical calcium phosphate aggregates after soaking in SBF, indicating that these materials are potentially bioactive. The key difference between their cell behaviour is the chemical nature and structural features of the glass fillers, responsible for the dissolution rate and chemical environment around the cells. The CSi composite demonstrated an inductive effect on the proliferation of MG63 and human bone marrow cells, and stimulated specific metabolic activities such as ALP activity and matrix mineralization suggesting that this composite may have a stimulatory effect on bone formation in vivo. The CB composite produced a certain inhibition of cell proliferation, probably due to the excessive presence of its ionic dissolution products in culture medium. The use of more dynamic cell culture conditions is expected to alleviate the observed deleterious effect on cells. Composites filled with lower percentage of borate glass may have better cellular behaviour, due to an expected decrease of ionic concentrations of the glass dissolution products. The comparative study of CSi and CB composites evidenced the high performance of CSi regarding bioactivity and biocompatibility and clearly indicated that, with respect to CB composite further and new experiments are required and a more in depth study should be made in order to assess its performance in biomedical applications.

Acknowledgements

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CHAPTER 3

PROPERTIES AND OSTEOBLASTIC CYTOCOMPATIBILITY OF SELF-CURING ACRYLIC CEMENTS MODIFIED BY GLASS FILLERS

ABSTRACT

New formulations of surgical PMMA-based bone cements with better properties than those commercially available were attempted. Materials filled with a silicate glass (MSi) and a borate glass (MB) were developed and compared in terms of their in vitro behaviour. The effect of proportion (0, 30, 40 and 50 wt%) and composition of filler on the curing parameters, residual monomer, water uptake, weight loss, bioactivity, mechanical properties (bending and compression) and osteoblastic cytocompatibility was evaluated. The addition of bioactive glass filler exhibited significant improvements in the curing parameters and in the mechanical properties of the cements. The most relevant results were obtained for the lower filler concentration (30 wt.%) a maximum flexural strength of 40.4 MPa for MB3 and a maximum compressive strength of 95.7 MPa for MSi3. On what concerns the bioactivity, the formation of the apatite layer was more effective for cements with higher glass content as expected. Regarding the biological assessment, the incorporation of the silicate glass significantly improved osteoblastic cytocompatibility, whereas the presence of the borate glass resulted in a poor cell response. Nevertheless it was demonstrated that the surviving cells on the MB surface were in a more differentiated stage compared to those growing over non-filled PMMA. Results suggest that the developed formulations offer a high range of properties that might be interesting for their use as self-curing cements.

This chapter is based on the following publication:

Lopes PP, Garcia MP, Fernandes MH, Fernandes MHV. Properties and osteoblastic cytocompatibility of self-curing acrylic cements modified by glass fillers. *Journal of Biomaterials Applications*, submitted.

INTRODUCTION

PMMA-based bone cements occupy a distinctive place in the hierarchy of synthetic biomaterials, because they are the only materials currently used for anchoring the prosthesis in orthopaedic surgery, being an alternative to non-cemented techniques [1].

The history of cemented joint arthroplasty begins with Charnley who described a totally new surgical procedure in 1958 [2]. Nowadays, acrylic bone cements are used in more than 90% of total hip surgeries in developed countries [3]. The clinical success rate of implanted cemented arthroplasties, especially those of the hip and knee in patients aged over 50 years, is very high, averaging at least 90% after 15 years [1].

The function of PMMA bone cements is to fill the space between the prosthesis and the bone, forming a mechanical bond with the surfaces, and transmitting the applied force and body weight uniformly to the tissue, working as a load bearing material [4]. Although universally used for many years PMMA bone cements are beset with a number of drawbacks that limit their performance such as non-bone-bonding capability, relatively low mechanical strength, release of unreacted monomer and high curing temperatures. These problems can cause serious complications *in vivo*, such as necrosis of the surrounding tissues and even loosening of the implant [5-7].

Among the attempts made to improve the properties of the bone cements, one can find the incorporation of a bioactive filler, promoting bioactivity, i.e. bone growth around the implant, resulting in increased longevity of the prosthesis [8]. However, it has been previously reported that the addition of a bioactive filler is limited due to the detrimental effect on the mechanical properties of the cement. The effect of filler is not clear, as it depends on a number of factors, such as the type of matrix and filler as well as their concentration [9, 10]. Vallo et al, showed that a maximum of 15 wt% HA (hydroxyapatite) [11] and 50 wt% of glass particles [8] can be incorporated in a cement to increase flexural modulus and fracture toughness. Lopes et al, verified that the addition of 30 wt.% glass, referring to the wt.% of the solid component, to a PMMA-co-EHA matrix resulted in significant increases in flexural strength and elastic modulus, thus enhancing the mechanical performance of the material [5]. Harper et al, reported that the addition of HA up to 40 wt% to a poly(ethyl methacrylate)-based bone cement increased both the flexural strength and modulus [12].

The N,N-dimethyl-4-toluidine (DMT) is currently used as activator in the curing of commercially available formulations of acrylic bone cements. *In vitro* studies on the toxicity produced by this activator demonstrated that it causes a delay on the cell replication when it is exposed to osteoblastic cell cultures [13]. Other undesirable characteristics of this compound include high toxicity, possible carcinogenic effects, chromosome-damaging, and inhibition of protein synthesis [14, 15]. To overcome these drawbacks alternative compounds have been

suggested. The application of 4,4-bis (dimethylamino) benzydrol has been recently studied giving rise to cured materials with improved biocompatibility [16], which was attributed not only to the reduced cytotoxicity of this new activator but also to its antiseptic properties [13].

The present study reports the preparation of cements with a low toxicity activator and the modification of the respective solid phase through the addition of two bioactive glasses (a silicate glass and a borate glass). The aim of the incorporation of the glass fillers was to improve the mechanical properties and to induce bioactivity on the system. It has been shown in previous works by the authors of this study that PMMA-based composite filled with the same glasses compositions (thermally cured composite) exhibited a fast formation of apatite in simulated body fluid (SBF), indicating the suitability of these materials for bone repair [17, 18]. The effect of the glass filler on the curing parameters, residual monomer, water uptake and weight loss was also investigated. In addition, the biological performance of the prepared cements was assessed with human osteoblastic bone marrow cell cultures.

MATERIALS AND METHODS

Cement preparation

New formulations of bone cements were prepared from a solid and a liquid phase by free radical polymerization. Methyl methacrylate (MMA monomer, Aldrich) was mixed with 4,4-bis (dimethylamino) benzydrol (Aldrich) as an accelerator/activator of the polymerization, resulting in the liquid phase. The solid phase was constituted by PMMA polymer (molecular weight of 120,000 and mean particle size of 100 μm , Aldrich), glass particles and benzoyl peroxide (BPO, Merck) as initiator. Two different glass compositions (Table 1) were used in this work, a silicate-based glass and a borate-based glass.

Table 1: Glass composition (mol%), specific surface area and density.

Glass	CaO	P ₂ O ₅	MgO	SiO ₂	B ₂ O ₃	SSA (m ² /g)	Density (g/cm ³)
Silicate	38,0	12.7	24.8	24.5		0.030	2.91
Borate	38,0	12.7	24.8		24.5	0.029	2.69

The glasses were prepared through the classic melt-quenching method following a procedure described in Lopes PP [17, 18]. The specific surface area (SSA) was measured following the BET (Brunauer–Emmett–Teller) method (Qyantasorb, QuantaChrome) and helium pycnometry (Micromeritics, Accupyc 1330) was used to determine the glass powder density. The solid and

liquid phases were manually mixed with a glass bar until the mixture became dough with a high viscosity. Then the dough was placed into a poly(tetrafluoroethylene) (PTFE) mould and cured at room temperature. The formulations were prepared by varying the composition of the solid phase, replacing 0, 30, 40 or 50 wt.% of the PMMA by the bioactive glasses. The final nominal composition of the cements is presented in Table 2, where PMMA refers to 0% glass, MSi3 and MB3 to 30%, MSi4 and MB4 to 40% and MSi5 and MB5 to 50% glass, in relation to the wt.% of the solid phase. The solid:liquid ratio employed was 1.75:1, with the initiator/activator in a molar ratio of 1.3. Some pores were observed in the cured cements which may be attributed to entrapped air during mixing and monomer evaporation over polymerization. When MMA polymerizes the air bubbles are embedded and covered by the PMMA matrix, which also encloses the bioactive glass particles.

Table 2: Composition of the solid and liquid phases of the developed cement (wt%).

Samples	Solid Phase			Liquid Phase
	PMMA	GSi	GB	MMA
PMMA	63.6			36.4
MSi3	44.6	19		36.4
MSi4	38.6	25		36.4
MSi5	31.8	31.8		36.4
MB3	44.6		19	36.4
MB4	38.6		25	36.4
MB5	31.8		31.8	36.4

Determination of curing parameters

Both cement components were mixed together in a small PTFE beaker for 1 min, and approximately 6 g of the obtained dough was then placed into a cylindrical mould at room temperature. The exothermic polymerization temperature was measured using a digital thermometer inserted in the curing mass at approximately 3 mm from the bottom of the tube, and was recorded every 10 seconds. Time was measured with a chronometer from the onset of mixing the powder with the liquid. Setting time (t_{set}) was calculated as the time at which the temperature of the mass corresponded to the sum of the ambient temperature (T_{amb}) and the maximum temperature (T_{max}) divided by two.

$$t_{set} \rightarrow T = \left(\frac{T_{max} + T_{amb}}{2} \right) \quad (1)$$

The measurements were done in triplicate according to ISO 5833 standards for acrylic resins by recording the polymerization temperature–time profiles [19].

Residual monomer content

The residual monomer content was measured by means of ¹H NMR (proton nuclear magnetic resonance) spectroscopy with a BRUKER AVANCE 300 Spectrometer operating at 300 MHz at room temperature. The samples were dissolved using deuterate chloroform as solvent and tetramethylsilane (TMS 1 vol.%) as the internal reference. The percentage of monomer moles present in the cured cement sample (%*Mr*) was calculated using the following expression:

$$Mr = \left(1.5 \times \frac{A_v}{A_m} \right) \times 100 \quad (2)$$

where *A_v* and *A_m* stand for the area of vinyl and methoxyl signals, respectively and 1.5 is a factor relating the number of protons of the methoxyl group (three) to those in the vinyl region (two). All the values were the average of three replicates [20, 21].

In vitro bioactivity

Specimens of 5x5x3 mm were mounted vertically and soaked in simulated body fluid (SBF) at physiological conditions of temperature 37 °C and pH 7.4. The SBF solution was prepared according to the formulation of Kokubo and Takadama [22], with ion concentrations nearly equal to those of human blood plasma. This solution was previously filtered through a Milipore 0.22 µm system and a constant specimen surface area to solution volume ratio of 0.1 cm⁻¹ was used. The materials were soaked for periods of 3, 7, 14 and 21 days.

Mechanical Properties

The various composites were tested on bending and compression. All samples were prepared in the same way to avoid the eventual influence of the preparation technique upon mechanical properties. Bending test specimens were produced by cutting the original composite slabs into beams of 40 mm x 5 mm x 4 mm using a band saw (Struers Secotom-10). Three-point bending tests were performed at room temperature in a Bose/Electro Force 3400 machine at a crosshead speed of 1 mm/min [6, 23]. Six samples of each composition were tested. The strength (σ_B) and the flexural modulus (E_B) were calculated using the standard formulae [24]. The E data were extracted from the initial linear portion of the load - displacement curve.

$$\sigma_B = \frac{3 \times F \times L}{2 \times b \times h^2} \quad (3)$$

$$E_B = \frac{L^3 \times \Delta F}{4 \times b \times h^3 \times \Delta y} \quad (4)$$

where F is the highest load of the load –displacement curve, L is the distance between end supports, b is the width, h is the height of the specimen, ΔF and Δy are the gradient of load and displacement, respectively, of the initial straight-line portion of the curve.

In compression tests, cylindrical samples (diameter 6 mm and height 12 mm) were deformed at speed of 20 mm/min. This mechanical assay was also carried out at room temperature in a Bose/Electro Force 3400 machine. For each set of composition 5 samples were tested. The strength (σ_C) and the flexural modulus (E_C) were calculated using the relationship:

$$\sigma_C = \frac{F}{A} \quad (5)$$

$$E_C = \frac{\Delta F \times h}{\Delta y \times A} \quad (6)$$

where F is the load applied just before crushing, A is the initial cross-sectional area, h is the height of the sample [24].

Water uptake and weight loss

These parameters of the self-curing materials were evaluated under simulated physiological conditions. The specimens were soaked in Phosphate Buffered Saline (PBS) at pH 7.4 and maintained at temperature 37 °C. A constant specimen surface area to solution volume ratio of 0.1 cm⁻¹ was used. At appropriate times, 3, 7, 14, 21, 28 and 60 days, the samples were rinsed with ultrapure water, blotted on filter paper to remove surface solution/water, and immediately weighed. Water uptake (WU) and weight loss (WL) were calculated using the following equations:

$$WU = \left(\frac{m_t - m_o}{m_o} \right) \times 100 \quad (7)$$

$$WL = \left(\frac{m_{f,t} - m_o}{m_o} \right) \times 100 \quad (8)$$

where m_t stands for the mass of the specimen at time t (days), m_o is the mass prior to immersion and $m_{f,t}$ is the final mass of the specimen kept in the oven until constant mass after t days of immersion in the PBS [25, 26].

Osteoblastic cytocompatibility

Cytocompatibility studies were performed with human osteoblastic cell cultures. Human bone marrow, obtained from orthopaedic surgery procedures, after patient informed consent, was cultured in α -Minimal Essential Medium (α -MEM) containing 10% fetal bovine serum, 50 μ g/ml

ascorbic acid, 100 IU/ml penicillin, 2.5 µg/ml streptomycin and 2.5 µg/ml fungizone, at 37 °C in a humidified atmosphere of 5% CO₂ in air. Primary cultures were maintained until near confluence (10–15 days) and, at this stage, adherent cells were enzymatically released (trypsin–EDTA solution). First-passage cells were seeded at a density of 2×10^4 cell/cm² over the surface of the cements. Seeded cements were cultured for 21 days in the presence of 50 µg/ml ascorbic acid, 10mM β-glycerophosphate and 10 nM dexamethasone. Colonized material samples were evaluated for cell viability/proliferation, alkaline phosphatase (ALP) activity and were observed by scanning electron microscopy (SEM) at days 7, 14 and 21, to evaluate cell morphology, pattern of cell growth and matrix mineralization.

Cell viability/proliferation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cell viability/proliferation. At days 7, 14 and 21, colonized materials were incubated with MTT, during the last 4 hours of the culture time tested. The formazan salts were dissolved with dimethylsulphoxide and the absorbance was measured at 492 nm in a ELISA reader (Synergy HT, Biotek). Three replicates were set up at each condition. Results were compared in terms of macroscopic surface area and expressed as Acm^{-2} .

Alkaline phosphatase activity

The colonized materials were treated with 0.1% triton in water (to lyse the cell layer) and the cell lysates were evaluated for ALP activity and total protein content. ALP was assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, 30 min at 37 °C, and colorimetric determination of the product (p-nitrophenol) at $\lambda=405$ nm. Enzyme activity was normalized by total protein content (determined by Lowry method). Results are expressed in nanomoles of p-nitrophenol produced per min per µg of protein ($\text{nmol} \cdot \text{min}^{-1} / \mu\text{g protein}$).

Scanning electron microscopy

For SEM observation, materials samples were fixed (1.5% glutaraldehyde in 0.14M sodium cacodylate buffer, pH 7.3, 10 min), dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analysed in a JEOL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDS) microanalysis capability (voyager XRMA System, Noran Instruments).

Statistical analysis

Statistical significance between groups was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison test post-analysis to evaluate statistical differences among samples. All values are expressed as mean and SD. A *p*-value below 0.05 was considered significant.

RESULTS AND DISCUSSIONS

Curing Parameters

In all cases, the maximum temperature of the composites containing bioactive glass was lower than that of the PMMA formulation used as reference. The incorporation of higher percentages of glass increased the setting time and decreased the maximum temperature Figure 1.

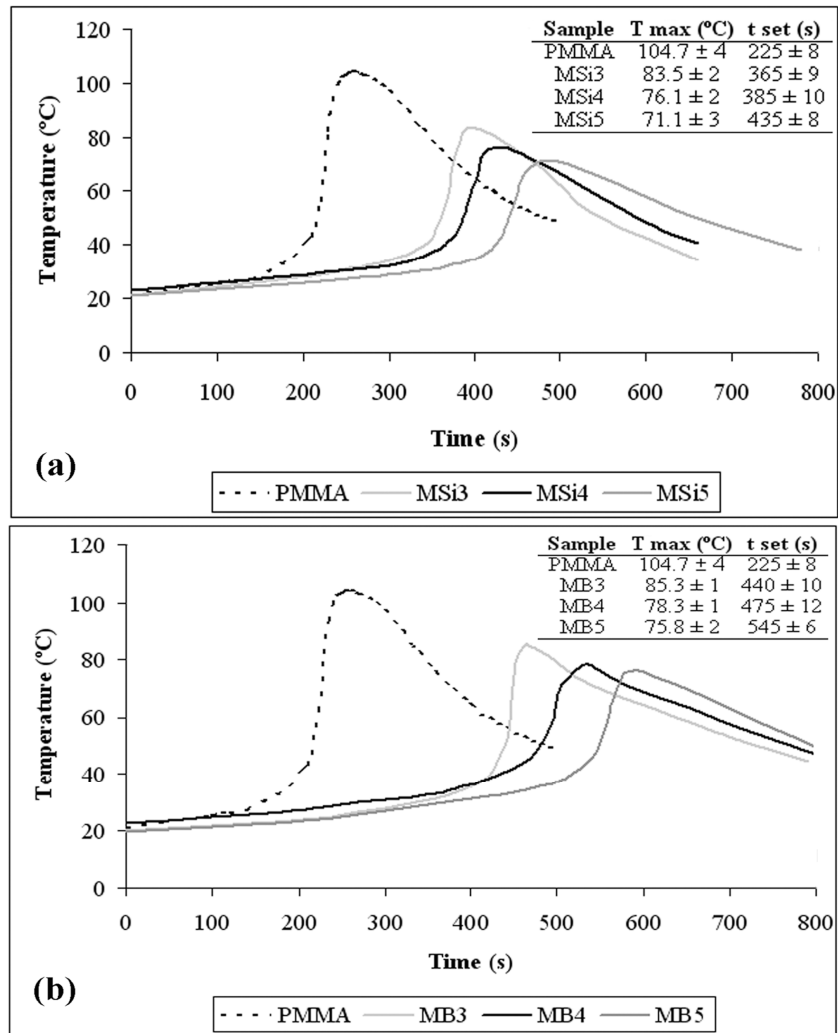


Figure 1: Curing parameters for cements filled with silicate glass (a) and cements filled with borate glass (b).

One way ANOVA indicated that the values obtained for the curing parameters of the different composites were significantly different ($p < 0.05$). To explore the differences among the means, Tukey's multiple comparisons were performed. The results showed that for the setting time, there is no statistical difference between the groups MSi5 and MB3, and MSi4 and MSi3. Regarding the maximum temperature, the mean of MSi3 and MB3 differs from the mean of the other groups and a significant difference was also detected between MSi5 and MB4. With respect to the control (PMMA), all obtained values were significantly different.

The maximum temperature reached during the curing of bone cements is directly related to the amount of heat produced as a consequence of the polymerization reaction of the liquid phase. This temperature is lower when the glass is present, since a homogeneously distributed solid material in the cement dough may absorb some of the heat produced by the exothermic reaction of polymerization [27]. Moreover, it is known that during the mixing a partial dissolution of the PMMA beads in MMA monomer occurs, hence their polymer chains become available for free radical polymerization acting as an additional initiator and consequently affecting the reaction kinetics [28]. Thus decreasing the amount of these PMMA particles will retard the polymerization, increasing the setting time and possibly decreasing the maximum curing temperature. Regarding this, it is worth mentioning that the peak temperatures, recorded *in vitro*, do not correspond to those actually reached *in vivo*. Clinical tests showed significantly lower intraoperative peaks at the bone-cement interface due to the thin layer of required cement, the heat dissipation of the system via the implants and local blood circulation [29].

The values of curing parameters of the cement with different glass contents fulfilled standard requirements for the bone cement [30]. The composition of glass (silicate or borate glass) does not have much influence on the maximum temperature, and even the effect of the proportion is limited since there is no difference between compositions with 40 and 50% of the glass. The cement filled with borate glass showed a setting time higher than the cement filled with silicate glass for all proportions (30, 40 and 50%). This behaviour can be related with the lower density of borate glass, 2.69 g/cm^3 , compared to the silicate glass, 2.91 g/cm^3 , resulting in a higher volume, for the same wt.% in the composite and a similar surface area of the glasses (Table 1). The high volume of glass promotes an increase of the contact interface, i.e. higher volume of the particles will be wetted by the monomer becoming unavailable for the curing process and thus the glass particles act as an array of barriers to the polymerization, delaying the setting time [31].

Residual Monomer content

It is known that the polymerization reaction of MMA ceases before consuming the available monomer. A total conversion can never be reached due to the mobility of the monomer molecules that diminish dramatically with the increasing dough viscosity. Therefore an amount of 2–6% of residual monomer can be present in a cement matrix [29]. The %Mr content for the prepared formulations is shown in Figure 2. One way ANOVA showed that the obtained values were significantly different ($p < 0.05$), except between PMMA and MSi3, and MSi4 and MB4.

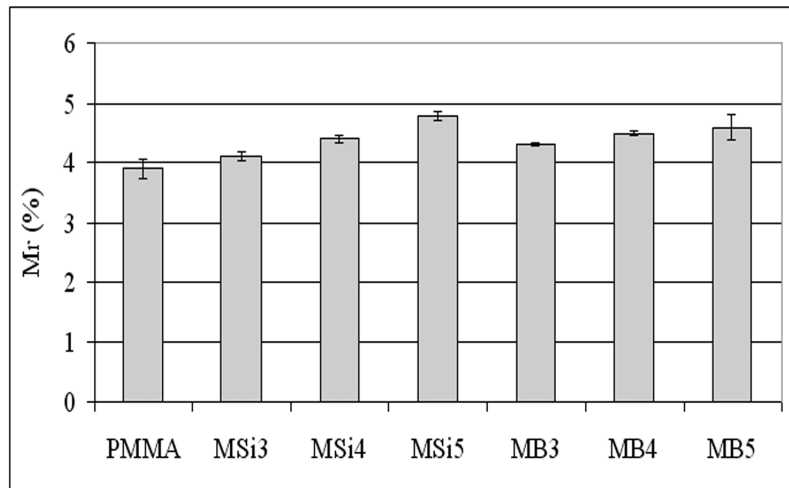


Figure 2: Residual monomer content for the studied cements.

The results revealed that the residual monomer content slightly increased with the addition of the glasses. It is believed that part of the monomer liquid can be immobilized on the filler surface, causing the increase of viscosity and consequently, the decrease of the polymerization rate with the filler content [32]. Moreover it was already mentioned that the presence of insoluble phases can interfere in the polymerization reaction, acting as an array of barriers. The mobility of the monomer can thus be greatly decreased leading to the increase of non-reacted monomer that remains trapped in the cement matrix. In the present study the values of the residual monomer content for all developed cements were lower than 5% which is within the acceptable limits for use as bone cements.

In vitro bioactivity

Figure 3 compares the precipitates formed on the surface of cements filled with silicate and borate glasses, later identified as calcium phosphate. It is clear that after soaking for 7 days spherical precipitates developed on all cements surfaces.

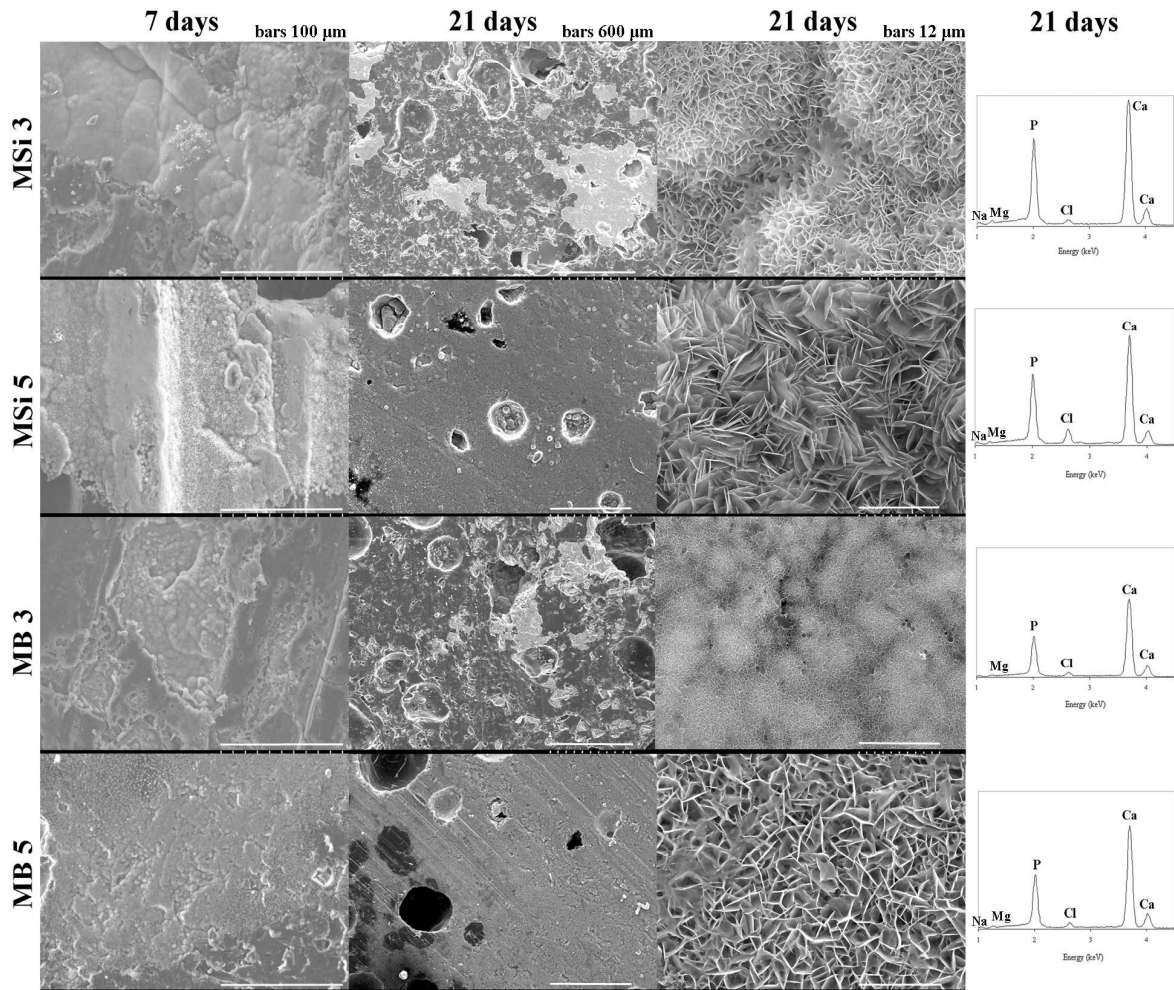


Figure 3: SEM micrographs of the cements surface after soaking in SBF and EDS spectra.

It can be seen from the micrographs that a dense layer was formed on the MSi5 and MB5 within 7 days, while the surface of MSi3 and MB3 were covered by sparse nuclei and few agglomerates. After 21 days, the layer completely covered MSi5 and MB5 surfaces, and its thickness increased. For MSi3 and MB3, their surfaces were not fully covered with the precipitates even after 21 days of immersion. The morphology of the precipitated layer on MSi5 and MB5 samples is different from that on MSi3 and MB3. This could be explained by the difference in the degree of crystallinity of the formed calcium phosphate, as it will be discussed below in detail based on the XRD patterns. The composition of the layer, on all cements, is similar and composed by Ca and P, with residual amounts of Na, Mg and Cl. After 21 days of immersion, the calculated Ca/P ratios for the precipitates were 1.64 for MSi3, 1.67 for MSi5, 1.55 for MB3 and 1.60 for MB5. As expected, the formation of the calcium phosphate layer was faster for cements with higher glass content.

For the cements filled with 40% glass after 21 days of immersion (results not shown), the MSi4 exhibited a behaviour similar to MSi5 developing a dense apatite layer, whereas for the MB4 the calcium phosphate formation was comparable to MB3 and a few precipitates were formed.

Further information on the structure of the layer was obtained by XRD, Figure 4. The patterns exhibited the characteristic peaks at $2\theta = 26$ and 32° attributable to apatite, reflections (0 0 2) and (2 1 1) respectively, for all cements investigated.

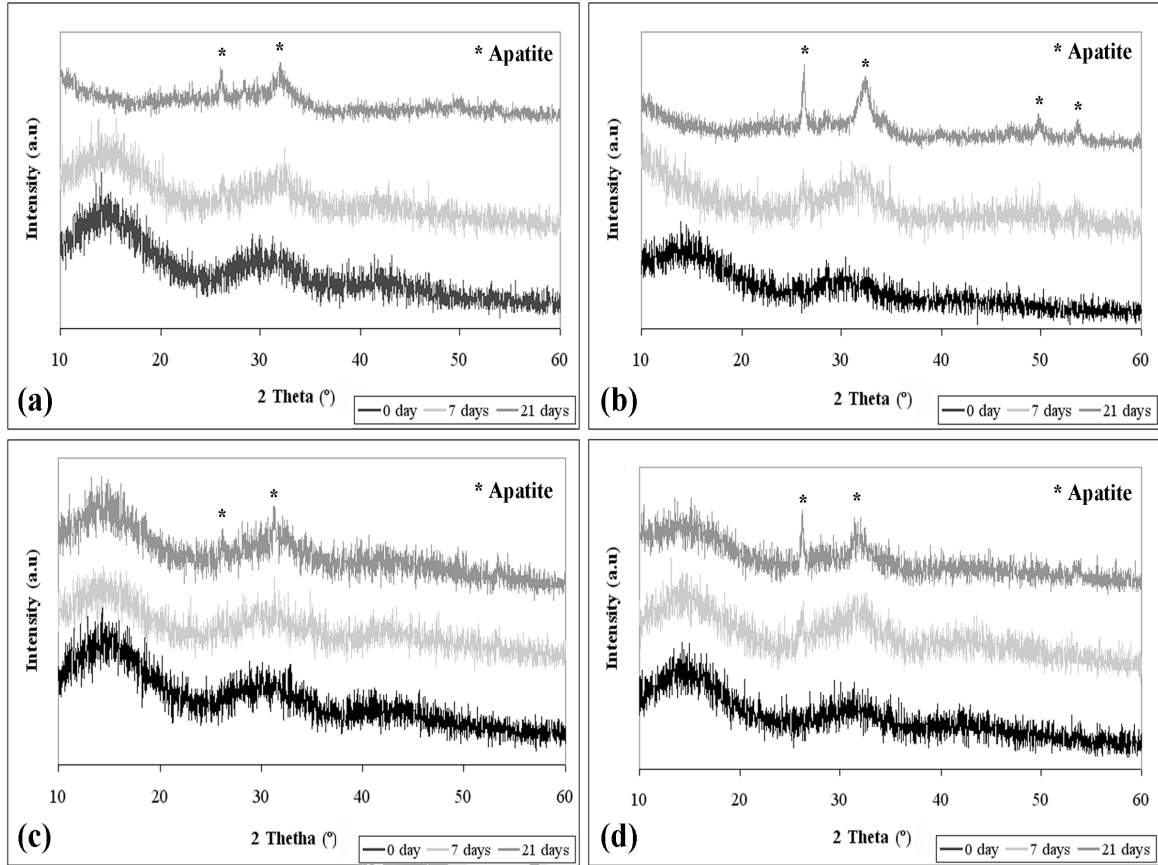


Figure 4: XRD patterns of surface cements MSi3 (a), MSi5 (b), MB3 (c) and MB5 (d).

For MSi5, other apatite peaks for $2\theta = 50^\circ$ and 53° – attributed to reflections (2 1 3) and (0 0 4) – became evident after 21 days of immersion [18]. The diffraction peaks were more defined and intense for MSi5 and MB5 when compared to those of MSi3 and MB3. In addition, the intensity of these peaks improves with increasing immersion time, indicating the enhancement of crystallinity of the apatite layer growing on the cement surface with time. XRD results also showed that the cements filled with higher glass concentration induced the precipitation in SBF of calcium phosphates with a higher degree of crystallinity.

The ICP curves for all formulations studied are illustrated in Figure 5. The variation of Ca and P concentration for both cements was due to the filler dissolution (increasing the concentration in the solution) followed by their consumption for the formation and growth of the calcium phosphate layer, resulting in the observed decrease in the concentrations of these ions in the solution at 21 days.

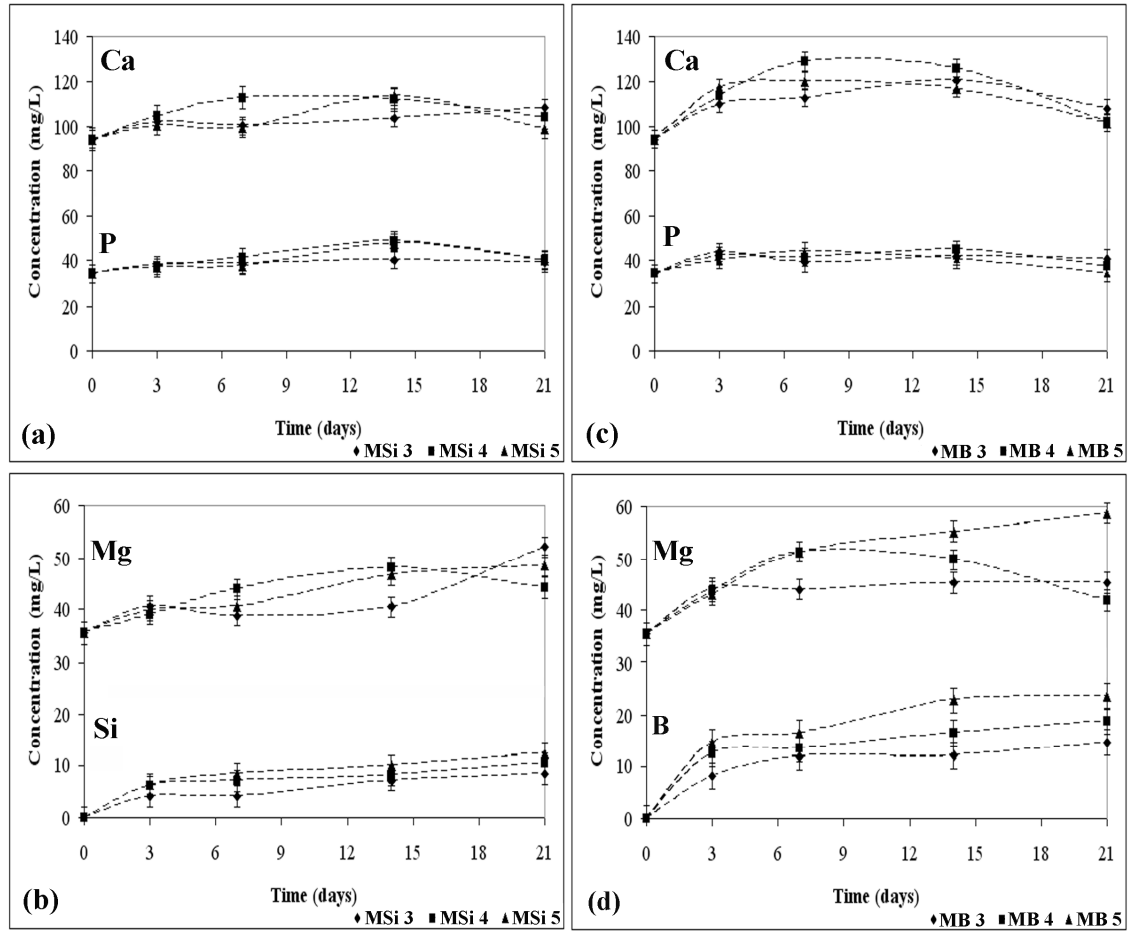


Figure 5: Variation of ionic concentration in SBF due to immersion of cements filled with a silicate glass (a, b) and cements filled with a borate glass (c, d). Dashed lines are to guide the eye.

This behaviour is more pronounced for cements filled with borate glass, especially in the case of Ca. The Mg and Si, for cements filled with silicate glass, and Mg and B for cement filled with borate glass, had their concentration continuously increasing as a result of glass dissolution and ionic exchange with the solution. The B concentration in solution is higher than the Si concentration. The less cohesive structure of the borate glass compared to the silicate glass can be responsible for the higher B release rate [33].

Structural and morphological observation of the surface layer indicated that cements with higher glass content induce higher calcium phosphate formation.

Mechanical Properties

The maximum strength and the elastic modulus, for bending and compression, of the samples are shown in Table 3. Glass filled cements exhibited improved mechanical properties when compared with the PMMA formulation used as reference. The most relevant results were obtained

for the lower filler concentration (30 wt.%) a maximum flexural strength of 40.4 MPa for MB3 and a maximum compressive strength of 95.7 MPa for MSi3.

Table 3: Mechanical properties of the samples.

Samples	Bending Properties		Compressive Properties	
	σ (Mpa)	E (Gpa)	σ (Mpa)	E (Gpa)
PMMA	24.3 ± 1.9	1.5 ± 0.1	85.5 ± 2.1	1.4 ± 0.1
MSi3	40.0 ± 4.8	2.1 ± 0.1	95.7 ± 2.2	1.8 ± 0.2
MSi4	34.8 ± 4.5	2.1 ± 0.2	92.3 ± 3.3	1.8 ± 0.2
MSi5	30.4 ± 4.4	2.1 ± 0.2	92.2 ± 3.3	1.9 ± 0.2
MB3	40.4 ± 2.9	2.2 ± 0.1	95.2 ± 3.2	1.9 ± 0.1
MB4	34.7 ± 2.2	2.1 ± 0.1	92.3 ± 1.5	1.8 ± 0.2
MB5	30.0 ± 2.6	2.1 ± 0.2	92.3 ± 2.0	1.9 ± 0.1

One-way ANOVA revealed that the differences in the bending and compressive strength and elastic modulus were significant ($p < 0.05$). Tukey's multiple comparison tests were used for pairwise comparisons among the group means. The bending strength of the cement filled with glass does not differ significantly ($p > 0.05$), except between cements MSi3 and MSi5/ MB5; and MB3 and MB5/MSi5 ($p < 0.05$). The elastic modulus (bending and compression) and compressive strength for the glass-reinforced composites were considered to be similar, but significantly different when compared to that of control (PMMA).

A 30 wt.% addition of glass brings improvements in the mechanical properties. However the increase in glass content to 50% can cause a heterogeneous distribution and agglomeration of the filler. When the filler starts aggregating, it behaves as a point of possible stress concentration, thereby weakening the cement and therefore the strength is reduced even if the filler is strong enough to increase the elastic modulus of the material [10, 34]. The same happens with the compressive properties.

The strength in composites depends on the strength of both the filler and the matrix, and the degree of adhesion between them. During the exothermic polymerization reaction, thermal stresses are generated due to the differences in the thermal expansion coefficients of the composite components. The shrinkage of the PMMA matrix is greater than of the glass particles according to the literature [35], resulting in a circumferential tensile stress adjacent to the glass. This leads to a weak bonding between the matrix and the filler and, hence, polymer–glass detachment can occur [27]. Previous studies have indicated that poor interface adhesion between the constituents of

composites can be responsible for their decreased mechanical properties [5, 11, 36]. The increase in glass content contributes to the aggravation of this problem.

Water uptake and weight loss

Water absorption may affect composite materials namely reducing their mechanical properties. Since dissolution of glass occurs in physiological medium, it was considered important to analyze both water uptake (WU) and weight loss (WL) in the developed cements. The evolution of the WU, WL and pH values with time is illustrated in Figure 6.

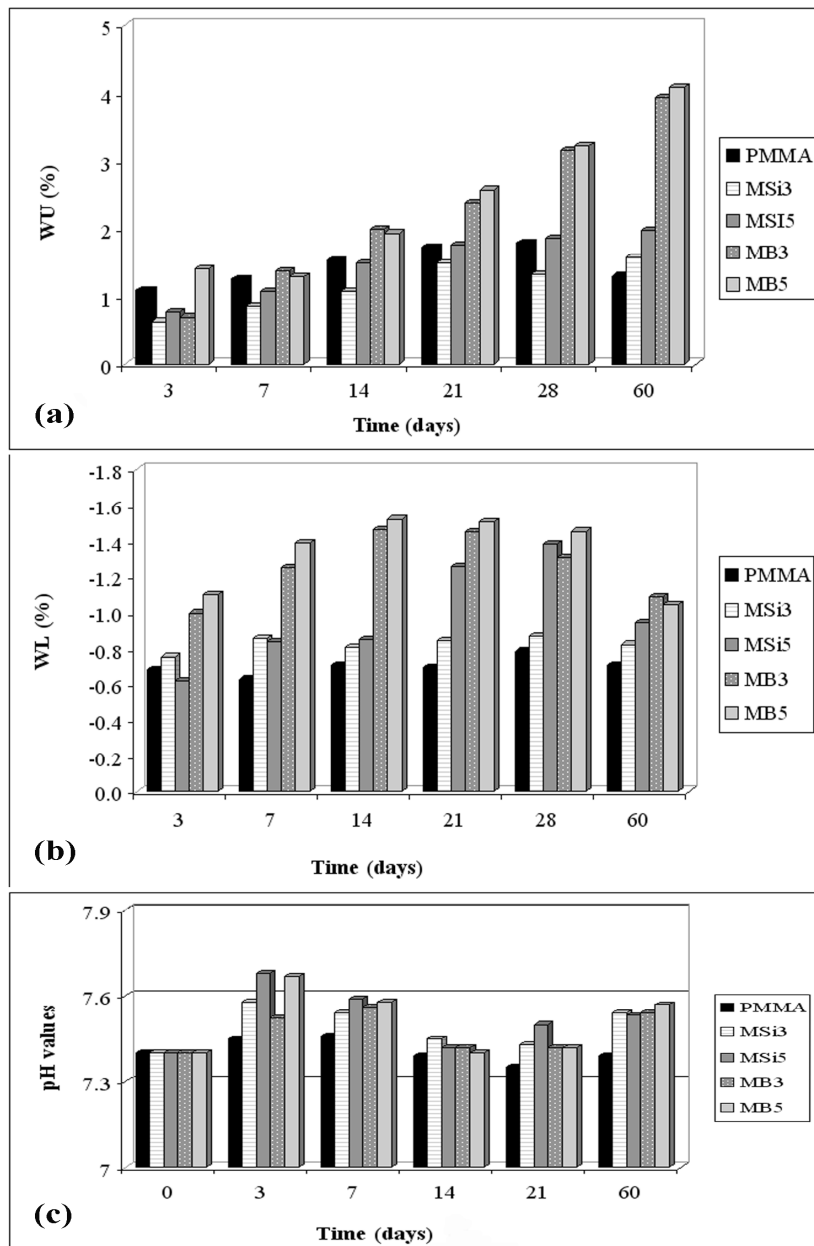


Figure 6: Water uptake (a), weight loss (b) and pH values (c) for the investigated formulations.

The data showed that the WU values for cements filled with borate glass were higher than control (PMMA), while for the cements filled with silicate glass the behaviour was the opposite, with the material showing a lower/similar water uptake. The amount of incorporated glass can also influence the water uptake. Overall, the results indicated that the compositions containing 50% of glass presented a higher WU capability than formulation with 30% glass during the same period. For cements with higher glass content the formation of void spaces around the glass particles are more likely to occur, allowing an easier penetration of water through these voids.

Bone cements are expected to maintain good mechanical properties after long periods of implantation, and consequently the water uptake should be constrained. It is known that commercial bone cements, due to their hydrophobic behaviour, present a low WU usually lower than 3 %, which can lead to a slight decrease in compressive and bending properties after 4 weeks in water at 37 °C [37]. In the formulations studied in the present work the highest obtained values for WU reached around 4% (Fig. 6a).

All the materials exhibited a small loss of weight during the test period (Fig. 6b). The obtained values were less than 1.6%, similar to those reported in other works, with HA-filled bone cements [38, 39]. In the present study, the cements filled with silicate glass presented lower WL, compared to the composites containing borate glass, which again is most probably due to the less cohesive structure of this glass [18, 33]. In general, for a given time, the formulations with higher WL correspond to the cements filled with higher glass filler amounts.

MSi3 showed a low WL during the 60 day immersion, but MSi5 exhibited an increase in WL after 14 days incubation, and at days 21 and 28, values were similar to those found in the materials containing borate glass. WL increased continuously in the B-glass composites up to day 14, being approximately constant until day 28. The MB5 showed the highest weight loss. The behaviour of borate glass composites correlate well with the observed WU, since the increase of water content would allow for more material to be leached out.

For a long incubation time (60 days), there was a decrease in WL, observed in both types of composites. The WL tests were carried out in PBS. It is known that the PBS is a buffer solution, isotonic, non-toxic, and does not contain Ca in its composition. However, it does not inhibit the calcium phosphate formation on the material surface. Thus it is probable that in the present case the ion exchange reactions between the glass and the solution is followed by the precipitation of a calcium phosphate layer, characteristic of the observed bioactive behaviour of the cements. The competition of both processes, degradation of the cement and calcium phosphate precipitation, is likely to explain the observed results.

The pH did not change significantly until the end of the test, with values lying between 7.3 and 7.6.

Osteoblastic cytocompatibility

Cell viability/proliferation

Human bone marrow cells, first subculture, were cultured over PMMA samples and the composite cements in conditions that favour osteoblastic differentiation [40, 41], and results regarding the proliferation/differentiation behaviour are shown in Figures 7 – 10.

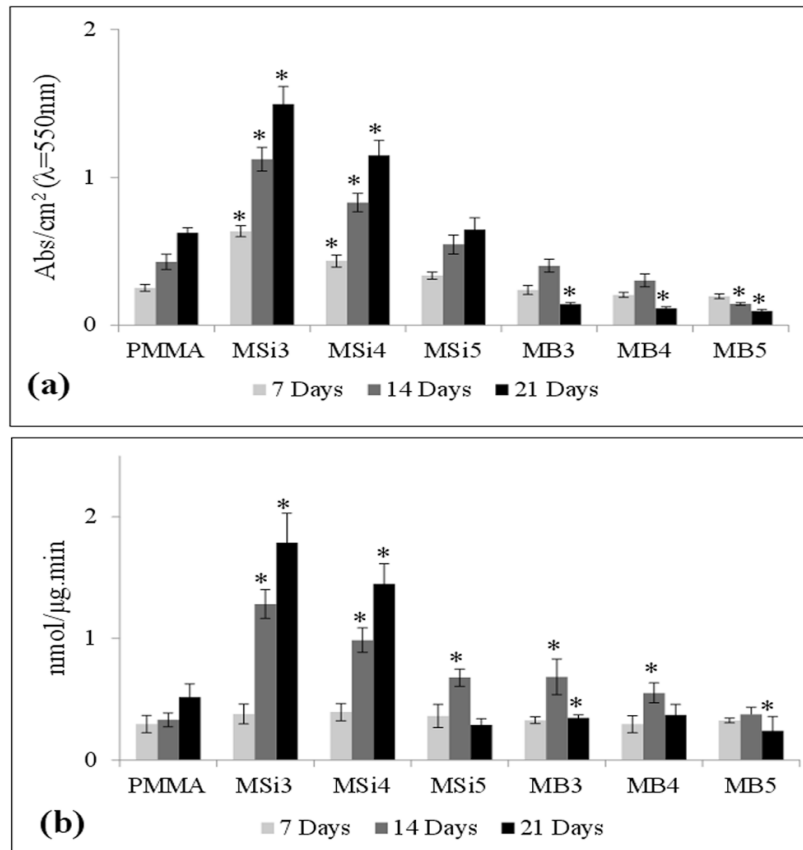


Figure 7: Cell viability/proliferation (A) and alkaline phosphatase activity (B) of human osteoblastic bone marrow cells cultured over PMMA and the glass-filled cements, for 21 days.*Significantly different from PMMA.

Colonized PMMA samples presented low values of cell viability/proliferation, which increased slowly throughout the 21-day culture period. The incorporation of Si glass resulted in an increase in the cell proliferation, but this positive effect decreased with the increase in the glass content. Compared to PMMA, compositions MSi3 and MSi4 presented significantly increased values (around 2 fold, at day 21). However, the composition MSi5 presented lower cell proliferation and, compared to PMMA, values were only slightly higher at days 7 and 14 and similar at day 21. The incorporation of B-glass did not improve the behaviour of PMMA regarding cell viability/proliferation. The composites presented lower values in the MTT assay and the negative

effect increased with the increase in the percentage of incorporated B-glass. The compositions MB3 and MB4 showed a low increase up to day 14 but, at day 21, MTT reduction values were very low. The composition MB5 exhibited low and progressively decreased values throughout the culture time. Results are shown in Figure 7A.

Osteoblastic differentiation

Cultures growing over PMMA presented low ALP activity at days 7 and 14, and a small increase at day 21. The compositions MSi3 and MSi4 showed significantly increased values, and enzyme activity also increased with culture time; at day 21, values were ~ 2 and 1.5 fold higher, respectively for MSi3 and MSi4, compared to PMMA. The composition MSi5 presented lower ALP activity, still an increase was noticed from day 7 to day 14, but afterwards values decreased. The composites MB3 and MB4 also showed an increase in ALP activity at day 14, being significantly higher than that on PMMA, but, at day 21, ALP activity was lower. MB5 displayed low values throughout the culture time.

ALP is an enzyme produced by osteoblastic cells and represents a frequently used early marker for osteogenic differentiation, as this enzyme has a determinant role in the mineralization of the extracellular collagenous matrix, by providing phosphate ions that, with calcium ions are used in the formation of the cell-mediated mineralized matrix [41]. The present results showed that the incorporation of Si-glass and B-glass in the PMMA matrix, in some of the tested concentrations, induced ALP activity, suggesting a positive effect in the osteoblast differentiation pathway. In the case of the Si composites, the ALP results were in agreement with those observed for cell proliferation, i.e. the compositions with a higher cell proliferation also presented a high increase in ALP activity. However, in the case of the B-composites, MB3 and MB4 showed low values for cell viability/proliferation but, still, an increase in ALP was noticed at day 14, suggesting that the surviving cells were in a more differentiated stage compared to those growing over PMMA.

SEM observation of the colonized materials was in line with the previous results, Figure 8. At day 14, PMMA showed few cells, but the surface was partially covered by a cell layer at day 21 (Fig. 8A,B). The cements containing the Si-glass presented a higher cell growth. At day 14, MSi3 and MSi4 (and also MSi5, at a lower extent) exhibited a cell layer partially covering the surface (Fig. 8C,E,G); cells showed an elongated morphology that tried to adapt to the underlying irregular surface, establishing cell-to-cell contacts. At day 21, MSi3 and MSi4 were completely covered by a thick cell layer (Fig. 8D,F), however, over MSi5 only few cells were visible (Fig. 8H). Regarding the composites with B-glass, at day 14, MB3 and MB4 showed few cells, mainly located in small niches/clusters scattered over the material surface (Fig. 8I,K). However, at day 21, cells were rarely

seen (Fig. 8J,L). MB5 barely supported cell growth, and cells were not evident on SEM observation (Fig. 8M,N).

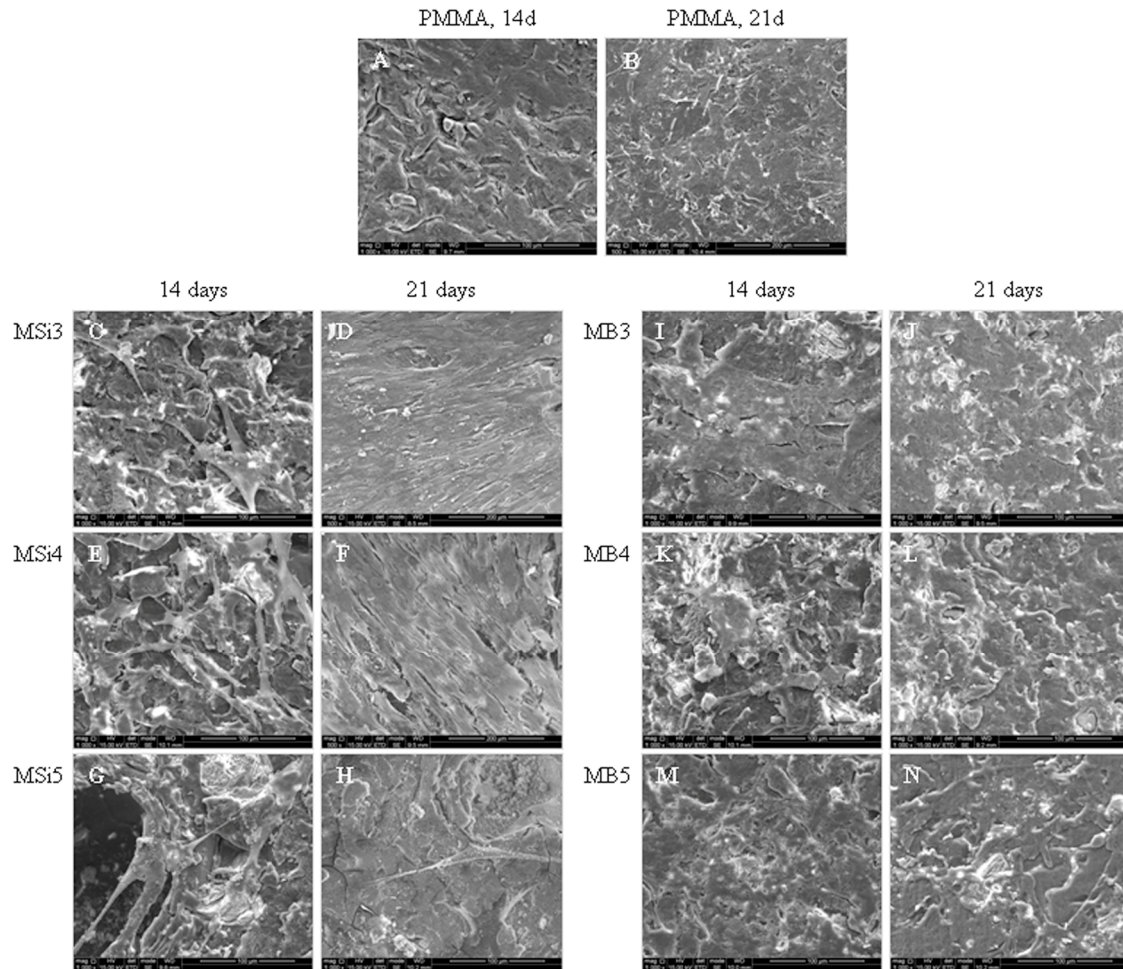


Figure 8: SEM observation of PMMA and the glass-filled cements colonized with human osteoblastic bone marrow cells, at days 14 and 21. Over PMMA, at day 14, cells were barely seen, but partially covered the material surface at day 21 (A, B). On the Si-glass compositions, cells were clearly seen at day 14 (C, E, G) and, at day 21, formation of a thick cell layer was observed over MSi3 and MSi4 (D, F), but few cells were visible on MSi5 (H). The B-glass cements presented significantly lower cell growth. Cells were visible at day 14 on MB3 and MB4 (I, K), mostly forming small cell clusters, but not over MB5 (M); at day 21, the presence of cells was not evident on the three composites (J, L, N). Bar = 100 μ m, except for B, D, F (200 μ m).

High amplification images, Figure 9, show that, at day 14, the compositions MSi3, MSi4 and even MSi5 presented mineralized globular deposits associated with the cell layer (Fig. 9A-C). At day 21, these globular formations were more abundant and organized on MSi3 and MSi4 (Fig. 9D,E), and X-Ray analysis showed well-defined Ca and P peaks (Fig. 9J). By contrast, MSi5, at day 21, exhibited a low number of cells, partially buried in a thick apatite layer that covered the material surface. This observation is in line with that reported in the bioactivity studies performed in SBF (Fig.3). The compositions MB3 and MB4 were also able to support the formation of cell-

mediated mineralized deposits within the small clusters/niches that were able to grow in localized areas of the surface (Fig. 9G,H). The X-Ray analysis also showed Ca and P peaks, although very discrete when compared to those seen on MSi3 and MSi4. It is worth mentioning that PMMA was not able to support matrix mineralization during the 21-day culture time. The globular structures associated with the cell layer, seen at day 21 (Fig. 9I), did not contain Ca and P peaks (Fig. 9L).

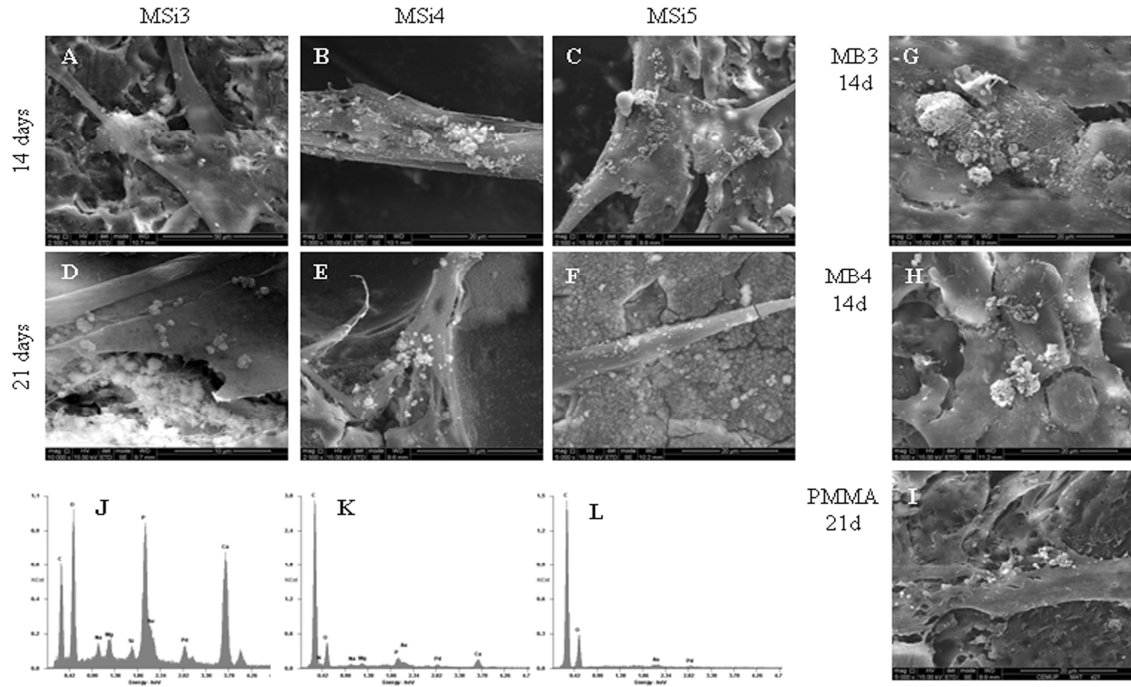


Figure 9: High magnification SEM images of PMMA and the glass-filled cements colonized with human osteoblastic bone marrow cells. The Si composites were able to form a mineralized matrix at day 14 (A – C) and MSi3 and MSi4 also showed a mineralized cell layer at day 21 (D, E); representative X-Ray spectrum of the globular mineral structures showed well evident Ca and P peaks (J). The MB3 and MB4 cements showed the formation of globular structures associated with the cell clusters at day 14 (G, H) and the x-Ray spectrum exhibited small Ca and P peaks (K). At day 21, PMMA also showed cell-associated globular structures (I), but they did not contain Ca and P peaks (L). A, C, E: Bar = 50 μ m; B, F-I: Bar = 20 μ m; D: Bar = 10 μ m.

As mentioned earlier, the prepared cements presented some pores scattered all over the surface, which on SEM observation appeared with a very smooth surface (Fig. 10A,D,E). SEM images of colonized MSi3 and MSi4 strongly suggested that cells did not colonize these locals. Representative images, Figure 10, shows a ring of cells growing around one of this pores and even forming bridges (Fig. 10A,D,E). Fig. 10B,C shows a more advanced stage, with the cells in the process of covering the pore and also some pores completed covered by a cell layer. Considering the protocol used in the preparation of these composites and that mentioned in materials and methods, the surface of these pores might be exposed PMMA that, together with the very smooth surface, probably explains the impaired cell growth within this structures.

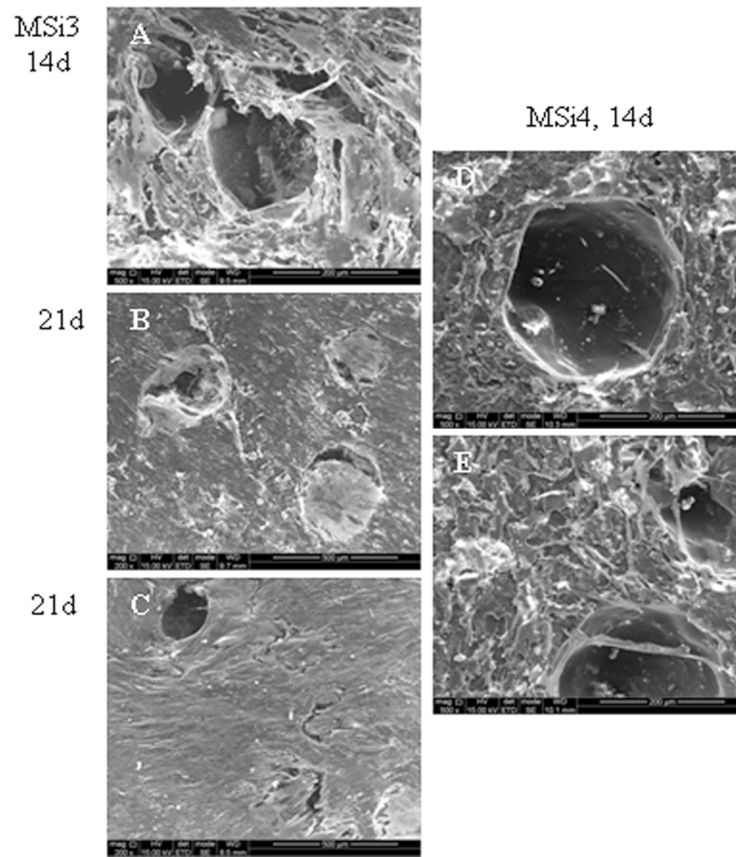


Figure 10: SEM images of MSi3 and MSi4 cements colonized with human osteoblastic bone marrow cells, showing that cells hardly colonized the pores present on the material surface. Images show the pores with a very smooth surface (A, D, E) and the cells growing around the pore (C, D), forming bridges (A, E) and covering the pores (B, C, arrows). A, D, E: Bar = 200 μm ; B, C: Bar = 500 μm .

Results showed that the incorporation of Si-glass in the PMMA structure improved cellular behaviour regarding viability/proliferation, ALP activity and matrix mineralization. However, this positive effect decreased with the increase in the percentage of incorporated glass. MSi3 presented a significant increase in these parameters, followed by MSi4, but MSi5 showed a modest effect. The immersion studies in SBF showed that these composites released Si ions (Fig. 5), and a similar behaviour is expected to occur in the culture medium. Silicon plays an important role in bone physiology, having a positive effect on osteoblasts [42]. The levels released ($\sim 5 - 10 \text{ mg/ml}$) are within a wide range (between 0.1 and 100 mg/l) reported to cause a dose-dependent increase in the proliferation and differentiation of osteoblastic cells and within the variable physiological range in humans [43]. Also, upon immersion in SBF, formation of an apatite layer was observed (Fig. 3), and this might also contribute to the improved osteoblastic cell response. However, the formation of this layer was faster on the cements with higher glass content due to a higher dissolution rate.

Accordingly, MSi5 developed a thick apatite layer and presented a high weight loss, suggesting a very dynamic surface with dissolution/ deposition events and a high ionic concentration in the cell environment, impairing cell survival. This was evidenced in SEM images, at day 21, showing mostly a thick apatite layer with some partially buried cells. Thus, the composites MSi3 and MSi4 probably meet a favourable combination of an appropriate apatite layer and Si release, resulting in significantly improved cell behaviour.

On the other hand, the incorporation of B-glass in the PMMA structure resulted in composites with a poor biological performance. Cell viability/proliferation was lower than that on PMMA. Some cell growth was noticed over MB3 and MB4, up to day 14, and ALP activity increased also at day 14 (attaining higher maximum levels, compared to PMMA). SEM images showed that cells were able to grow in small niches sparsely seen on the material surface. These niches, apparently, provided a protected environment allowing for a complete osteoblastic differentiation. The poor performance of the B-composites is most probably related to the less cohesive structure of borate glass, leading to a high degradation rate, as shown by the water uptake and weight loss studies (Fig. 6). As referred above, this originates highly dynamic ion exchange reactions, which is deleterious for cell growth. This behaviour was more evident in the composites with higher glass content, and, in line with this, MB5 barely supported cell growth. Also, it provides an explanation for the deterioration of the cell behaviour during the last week in all the B-glass compositions. The high degradation rate also contributes to a higher release of B ion (Fig. 5), which is reported to be toxic above a certain threshold value [44, 45], and, also, of the residual cytotoxic monomer present in the preparation.

In vivo, it is believed that the deleterious effect of a high degradation rate might be greatly attenuated. The continuous circulation of the body fluids would probably prevent an excessive ion concentration at the cell/material interface. Also, upon the implantation of a material in the bone tissue, there is a continuous availability of osteoblast progenitor cells that can adhere to the material surface when the appropriate sets of conditions are met.

CONCLUSIONS

The results suggest that the properties of the investigated cements are more dependent on the filler concentration than on the composition of the glass (silicate or borate glass). The addition of higher percentages of the glass promoted a diminishing of the exothermal effect on curing, reducing the peak of temperature, which is expected to contribute to the decrease of thermal necrosis of the surrounding tissue. All cements modified with glass reached higher mechanical properties than the PMMA matrix, although the most relevant results were obtained for the lower

filler concentration (30 wt%). Regarding the bioactive behaviour, the formation of the apatite layer was more effective for cements with higher glass content. The composition of the glasses was relevant to WL and WU measurements, since the cements filled with borate glass showed the highest weight loss and water uptake along the test. The performance of the cements filled with silicate glass was similar to the unfilled PMMA matrix, except for MSi5, at 21 and 28 days, for which the WL values were comparable to those of the cement filled with borate glass.

The Si-glass and the B-glass cements differed significantly in the elicited osteoblastic cell response. Incorporation of the silicate glass improved osteoblastic cytocompatibility, whereas the presence of the borate glass resulted in a poor cell response. For both types of composites, the cell response progressively deteriorates with the increase of the glass content. The less cohesive structure of the borate glass might be responsible for the increased degradation rate, the high B ion concentration in the physiological fluid and, eventually, favouring the monomer release, all probably contributing to a poor cell response.

Results suggest that the developed formulations exhibit a wide range of properties that might be interesting for their use as self-curing modified cements.

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CHAPTER 4

INFLUENCE OF IBUPROFEN ADDITION ON THE PROPERTIES OF A BIOACTIVE BONE CEMENT

ABSTRACT

Bioactive bone cements can promote bone growth and the formation of a strong chemical bond between the implant and bone tissue increasing the lifetime of the prosthesis. This study aims at synthesizing a new bioactive bone cement with different amounts of ibuprofen (5, 10 and 20 wt.%) using a lower toxicity activator, and investigating its in vitro release profile. The effect of ibuprofen (IB) on the setting parameters, residual monomer and bioactivity in synthetic plasma was also evaluated. It was verified that the different IB contents do not prevent the growth of calcium phosphate aggregates on composite surfaces, confirming that the cements are potentially bioactive. A relevant advantage of these formulations was a significant improvement in their curing parameters with increasing IB amount, associated to a reduction of the peak temperature and an extension of the setting time. The cements released about 20% of the total incorporated ibuprofen during 30 days test. This behaviour was attributed to the low solubility of this drug in aqueous media and was also related with the hydrophobic character of the polymer. Regarding the therapeutic concentration sufficient to suppress inflammation, the cement with 10% of ibuprofen achieved the required release rate for one week and the cement with 20% for two weeks.

This section is based on the following publication:

Lopes PP, Silva MS, Fernandes MHV. Influence of ibuprofen addition on the properties of a bioactive bone cement. *Biomedical Materials*, under revision.

INTRODUCTION

Bone cements are typical bioinert materials exhibiting as major limitation the lack of adhesion to bone, which may cause aseptic loosening and failure of the prosthesis in some cases [1, 2], owing to the formation of fibrous tissue around the cement that impedes the bone growth towards the surface [3]. In order to achieve better clinical results for fixation of orthopaedic implants bioactive PMMA bone cements have been developed showing a direct bond with living bone [4, 5] due to the precipitation of an apatite layer on their surface also found when in contact with a physiological medium [6]. It is known and currently accepted that this phenomenon is a reliable indication that, in vivo, bone growth will occur on the surface of the implanted material [7-10]. Thus the incorporation of a bioactive component such as a bioactive glass, into acrylic bone cement formulations seems to be the proper path towards the improvement of the interfacial cement-bone attachment.

Cemented orthopaedic implants are indwelling medical devices, intended for long-term presence in bone tissue. So they are at risk of infection or inflammation if a small amount of bacteria succeeds in colonising the foreign material [11]. In addition, microcracking may lead to PMMA-particle release, which can also induce local inflammation and osteolysis [12].

Although it is possible to use non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of inflammations, their applications are often restricted owing to side effects caused on the gastrointestinal tract resulting from the high oral doses [13-15]. Moreover, inflammatory conditions in bone tissue cause a reduced supply of blood and also of drugs transported via blood circulation. The potential side effects of systemic treatment and the relatively slow absorption (Bramlage and Goldis [16] report that peak plasma concentrations of ibuprofen are reached within about 1 to 2 hours after ingestion) can be avoided through local application, providing therapeutic drug concentrations only to intended targeted sites. Hence it is desirable to administer the drug locally with controlled dosage level, thus reducing the required drug concentration and potentially minimising its undesired secondary effects [17].

The addition of therapeutic agents to acrylic bone cements to assess its suitability as a drug delivery system began as early as 1969 [11]. Local delivery of NSAIDs from PMMA acrylic bone cement has been studied as a possible tool in the treatment of inflammations related to periodontitis [18]. Other studies demonstrated that bioactive acrylic bone cements loaded with anti-inflammatory/analgesic agent could be used as injectable formulations for minimally invasive vertebroplasty, showing some advantages over the PMMA. The results indicated a mild inflammatory reaction around the implanted material [19]. In this context, it appears as quite

pertinent and useful to investigate new cements that simultaneously exhibit controlled drug release and bioactive behaviour.

Ibuprofen is one of the most representative compounds of NSAIDs series. It is an analgesic, antipyretic and anti-inflammatory drug extensively used to treat bone diseases like rheumatoid arthritis, osteoarthritis and a number of other painful conditions [20]. It is known that ibuprofen reduces inflammation by the inhibition of the enzyme cyclooxygenase, halting prostaglandin synthesis and therefore diminishing the pain and swelling associated with the inflammatory process [21, 22]. This drug has a short half-life of about 2 hours, which is the period of time required for the concentration of the drug in the body to be reduced to exactly one-half of a given concentration, and a duration of action of 4-6 hours [23]. The therapeutic concentration of ibuprofen in plasma is usually in the range 20 to 30 mg/L, and a single oral dose of 400 mg produces a peak plasma concentration of 37 mg/L [24].

An important parameter to consider in the development of bone cement is the toxicity of the activator. In the recent years research has concentrated on developing an alternative to N,N-dimethyl-4-toluidine considered a highly toxic amine [25]. Lower toxicity activators, such as 4,4-bis-dimethylamino benzydrol have been studied, giving rise to systems with antiseptic properties and low toxicity that allow connective tissue to grow and attach to the material [26].

This study aims to prepare new formulations of self-curing acrylic bone cements containing bioactive glasses of the $3\text{CaO} \cdot \text{P}_2\text{O}_5\text{-MgO-SiO}_2$ system and to investigate the effect of ibuprofen proportion (5%, 10% and 20%) on its in vitro release profile, setting parameters, residual monomer content and bioactivity in simulated plasma. An activator of reduced toxicity was chosen for the preparation of the investigated cements. The observed in situ release of drug suggested that the ibuprofen-loaded cements can be appropriate to counteract the inflammatory response associated to the cement implantation.

MATERIALS AND METHODS

Materials

Methyl metacrylate (MMA monomer), PMMA beads (polymer), 4,4-bis (dimethylamino)-benzydrol (activator) and Ibuprofen were purchased from Aldrich. PMMA beads, with a molecular weight of 120,000, were milled in a rotor mill (Retsch ZM 200) to a mean particle size of 100 μm . Benzoyl peroxide (BPO, Merck) initiator and activator were used as received for the polymerization reaction.

A glass from the $3\text{CaO} \cdot \text{P}_2\text{O}_5\text{-MgO-SiO}_2$ system with the composition (mol%) 38% CaO, 12.7% P_2O_5 , 24.8% MgO and 24.5% SiO_2 was fabricated by the traditional melt quenching method. The

resultant glass frit was dry-milled (Retsch, RM100 Mortar Grinder Mill) to a powder with mean particle size of 10 μm , measured with a Coulter LS Particle Size Analyzer. The amorphous character of the glass was confirmed by X-ray diffraction (XRD, Rigaku Geigerflex Dmax-C with $\text{CuK}\alpha$ radiation).

Preparation of the cements

The preparation of the investigated bone cements was carried out by adding the solid components to the liquid components. The activator (4,4-bis (dimethylamino) benzydrol) was dissolved in the liquid phase and the initiator (benzoyl peroxide) was added to the solid phase. Both phases were manually mixed with a glass bar until the mixture became dough with a high viscosity. Then the dough was placed into a poly(tetrafluoroethylene) (PTFE) mould and cured at room temperature. Five formulations were prepared (Table 1) by varying the composition of the solid phase, replacing the PMMA by a bioactive glass and adding different amounts of ibuprofen (5, 10 and 20 wt.%). The solid:liquid ratio employed was 1.75:1, with the initiator/activator in a molar ratio of 1.3. The addition of ibuprofen was carried out during the preparation of the cement and not by soaking into a highly concentrated drug solution because the hydrophobic character of the matrix and the low porosity of the cement could hamper the proper incorporation of drug.

Table 1: Solid phase composition of the formulations produced (wt%)

Formulations	Solid Phase		
	PMMA	BG	IB
PMMA	100	-	-
IB0	50	50	-
IB5	45	50	5
IB10	40	50	10
IB20	30	50	20

Liquid phase constituted by MMA monomer.

The solid:liquid ratio employed was 1.75:1

Setting parameters

Both cement components were mixed together in a small PTFE beaker for 1 min and approximately 6 g of the dough was then placed into a glass tube in an oil bath at a temperature of 37 °C. The exothermic polymerization temperature was measured using a digital thermometer inserted in the curing mass at approximately 3 mm from the bottom of the tube, and was recorded every 10 seconds. Time was measured from the onset of mixing the powder with the liquid using a

chronometer. Setting time (t_{set}) was calculated as the time at which the temperature of the mass corresponded to the sum of the test temperature (37 °C) and the maximum temperature attained (T_{max}) divided by two. All the values were the average of at least three replicates. This procedure was based on earlier works [25, 27, 28].

Residual monomer content

The residual monomer content was measured by means of ¹H NMR (proton nuclear magnetic resonance) spectroscopy with a BRUKER AVANCE 300 Spectrometer operating at 300 MHz at room temperature. The samples were dissolved using deuterate chloroform as solvent and tetramethylsilane (TMS 1 vol.%) as the internal reference. The residual monomer in the cured cement was measured one month after the polymerization at room temperature. The percentage of monomer moles present in the sample (Mr) was calculated using the following expression:

$$Mr = \left(1.5 \times \frac{A_v}{A_m} \right) \times 100 \quad (1)$$

where A_v and A_m stand for the area of vinyl and methoxyl signals, respectively and 1.5 is a factor relating the number of protons in the vinyl region (two) to those of the methoxyl group (three) [29, 30]. All the values were the average of three replicates.

Assessment of in vitro bioactivity

The bioactivity of a material is regarded as its capability to bond to living bone through the precipitation of an apatite layer on its surface and this in vivo apatite formation can be reproduced in vitro through acellular tests using buffer solutions [8].

In a first stage, the in vitro bioactive behaviour of the prepared bone cements was studied in Phosphate Buffered Saline (PBS). The specimens were mounted vertically and soaked in PBS at physiological conditions of temperature 37 °C and pH 7.4. This solution was previously filtered through a Milipore 0.22 µm system and a constant specimen surface area to solution volume ratio of 0.1 cm⁻¹ was used. The materials were soaked for periods of 7, 14 and 21 days. After immersion the specimens were removed from the fluid and surface modifications were followed by X-ray diffraction (XRD) and scanning electron microscopy coupled with X-ray energy dispersive spectroscopy (SEM-EDS, Hitachi S-4100, Japan) at an acceleration voltage of 25 keV and beam current of 10 µA. EDS analyses were performed with a magnification of 500x on an analyzed area of 60 µm x 60 µm, to assess the evolution of the chemical composition of surface.

Then, in a second stage, the immersion tests were conducted in the same experimental conditions using Simulated Body Fluid (SBF) [8]. The cements were soaked for periods of 3, 7 and

14 days. The samples surfaces were observed by SEM-EDS and the fluid was analyzed by inductively coupled plasma spectroscopy (ICP, using a Jobin–Yvon JY70 Plus spectrometer).

In vitro drug release

The drug release study was carried out in PBS for periods up to 30 days and the IB release was followed by ultraviolet (UV) spectroscopy, using a Perkin-Elmer 554 spectrophotometer. The samples were soaked in 10 ml of solution at pH 7.4 and maintained at 37 °C. The solution was renewed after every analysis, following a protocol suggested in the literature [17], namely every 2 hours during the first day, once a day in the first week and every 2 days until the end of the test. The IB released to the buffer solution was measured at $\lambda = 264$ nm, and its corresponding concentration was calculated from a previously prepared calibration curve. The results were the average of four samples for each studied formulation.

The release mechanism of ibuprofen was further examined in order to better understand its liberation profile and the controlling key parameters. Drug release from a polymer system, following the Korsmeyer [31] and Peppas [32] model, can be described by the semi-empirical equation widely known as power law:

$$\frac{M_t}{M_\infty} = kt^n \quad (2)$$

where M_t stands for the drug released at time t , M_∞ is the quantity of drug released at infinite time, k is the kinetic constant and n is the release exponent, indicative of the drug-release mechanism. For cylindrical specimens, $n \leq 0.45$ means a Fickian diffusional release, $0.45 \leq n \leq 0.89$ an anomalous (non-Fickian) diffusion, and $n \geq 0.89$ the Case II relaxational release [20].

Statistical analysis

One-way ANOVA and multiple comparisons by the Tukey all pairwise approach were performed to determine the statistical significance ($p < 0.05$) of the differences among the groups.

RESULTS AND DISCUSSIONS

Curing Parameters

The temperature–time curves during the polymerization and the values of curing parameters of the formulations developed are illustrated in Figure 1. The results are shown as the arithmetic mean and the standard deviation (\pm SD). One way ANOVA test indicated that the values obtained for the maximum temperature (T_{\max}) and setting time (t_{set}) were significantly different ($p < 0.05$). The addition of bioactive glass and ibuprofen to the bone cement promoted significant improvements in

the curing parameters, usually associated with decreases in T_{\max} and increases in t_{set} inducing a slower curing process.

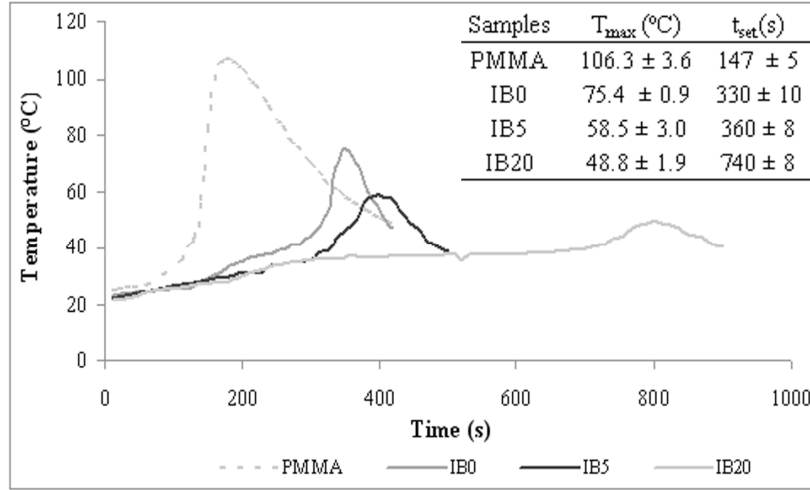


Figure 1: Setting parameters for the investigated formulations.

This behaviour was expected since the presence of a homogeneously distributed solid material in the cement dough may absorb some of the heat involved in the exothermic reaction of polymerization, causing a decrease in the temperature of the cement [33]. Moreover, the PMMA particles may contain peroxides and act as an additional initiator, affecting the reaction kinetics and contributing to the increase in the system viscosity [34]. Thus decreasing the amount of these particles will retard the polymerization, increasing the setting time and decreasing the maximum curing temperature.

The values of curing parameters for different ibuprofen contents fulfilled standard requirements for acrylic bone cements, which constrain the maximum temperature to 90 °C and the maximum setting time to 15 min (900 s), in accordance with ISO specifications [35].

Residual Monomer content

During the curing of the cement a substantial increase in the viscosity of the mixture takes place due to a partial dissolution of the PMMA in its monomer. The mobility of the monomer is greatly decreased by the increase in viscosity, which always causes the presence of 2–6% of residual monomer in a cement matrix [36, 37]. This percentage may be decreased with time up to 1-2% and then remain the same for years [38]. Haas et al measured the residual MMA monomer content to be 3.3% after 1h, 2.7% after 20h and 2.4% after 215 days under storage in an ambient air environment, indicating that the variation was negligible between 20h and 215 days [39].

In the present study, the residual monomer in the cured cement was measured one month after the polymerization at room temperature. The values are depicted in Figure 2, being evident that the residual monomer content lies in the range 0.9 – 1.75%. One way ANOVA test indicated that the obtained values were significantly different ($p < 0.05$), except between IB5 and IB20, and IB10 and IB20. Although within acceptable limits for use as bone cements, showing that the polymerization reached high conversions, the values of %Mr demonstrate that the addition of ibuprofen to the cement increased the residual monomer content. The presence of an insoluble phase like ibuprofen can interfere in the polymerization reaction, thus the mobility of the monomer is greatly decreased and the process evolves more slowly, leading to the increase of non-reacted monomer that remains trapped within the cement matrix.

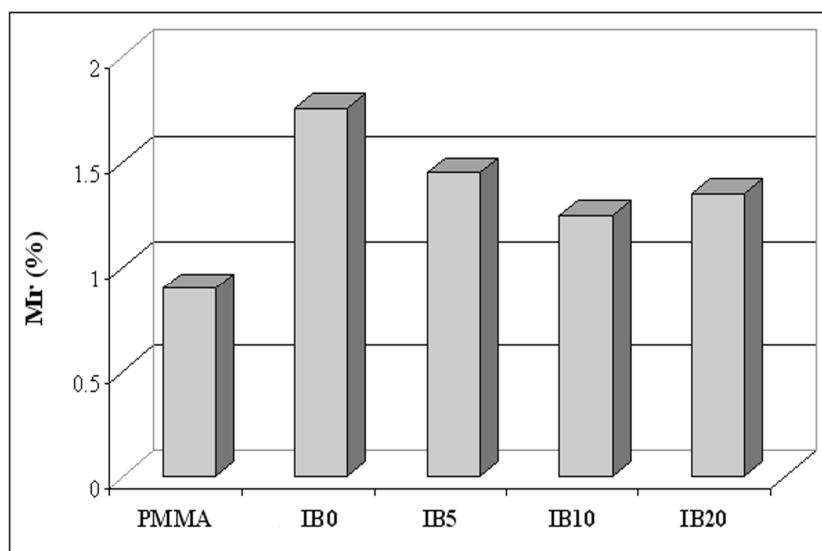


Figure 2: Residual monomer content for the studied cements, one month after polymerization.

In vitro bioactivity

The bioactivity test showed that for all ibuprofen contents the composites developed a calcium phosphate layer on its surface after immersion in a PBS solution. Micrographs of the composite surfaces are shown in Figure 3.

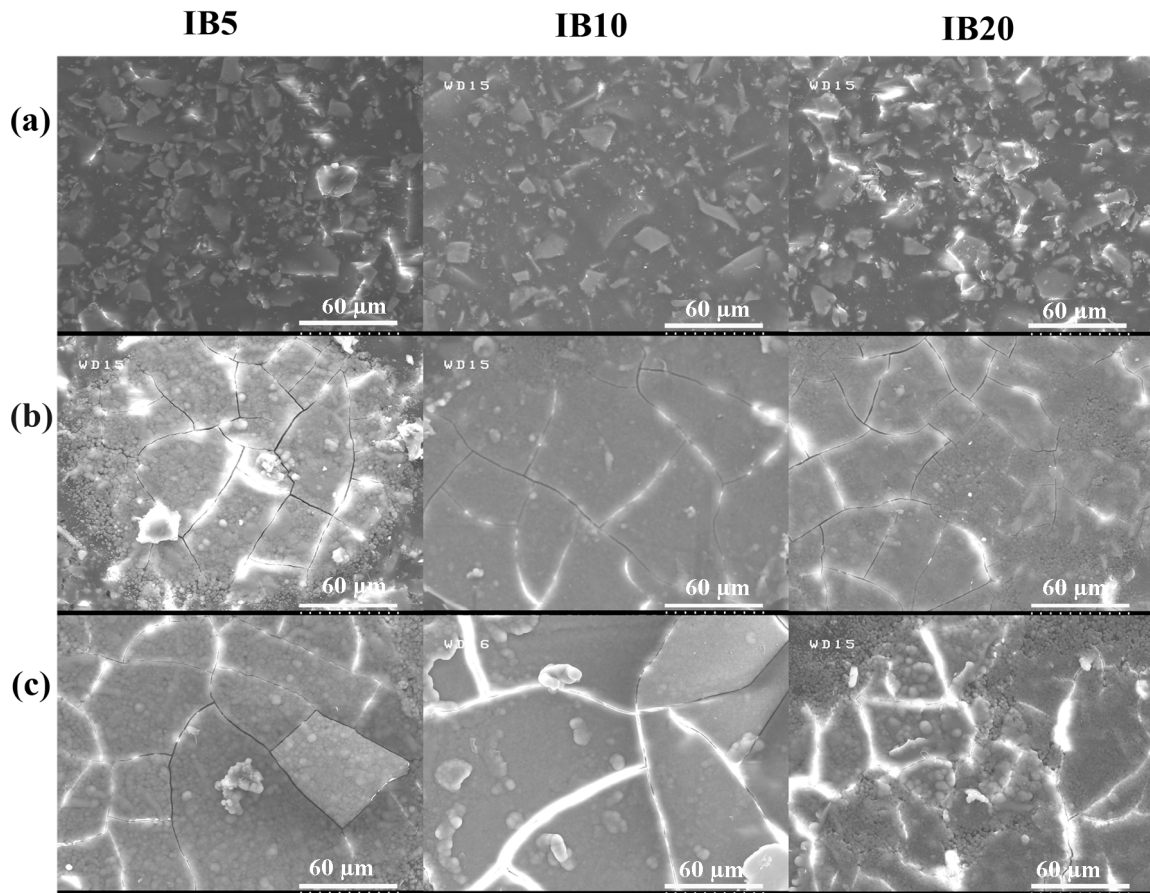


Figure 3: SEM micrographs of the bone cements surface (a) before immersion, (b) after 7 days and (c) after 21 days in PBS.

It is clear that after soaking for 7 days precipitates developed on the composite surface, although with undefined morphology probably due to the small amount, small size and low crystallinity of the formed precipitates. The crystallinity of the layer does not seem to be altered even for the higher immersion time, 21 days. This result was also confirmed by the XRD analysis shown in Figure 4.

The surface chemical analysis by EDS, for all cements is depicted in Figure 5. The obtained results for 0 day of immersion (indicated as Initial in figure) confirmed that the relation between elements that constitute the composites before immersion are in agreement with the respective ratio in the glass composition. After 7 days, the Si and Mg signal decreased and the composite surfaces exhibit a pronounced increase in the Ca and P signal, suggesting calcium phosphate deposition. The presence of Na and Cl was also detected for this soaking time. The sodium chloride could be incorporated into the calcium phosphate network due to their high concentrations in PBS. After 21 days a clear increase in Ca and P signal was found at the surface of all composites.

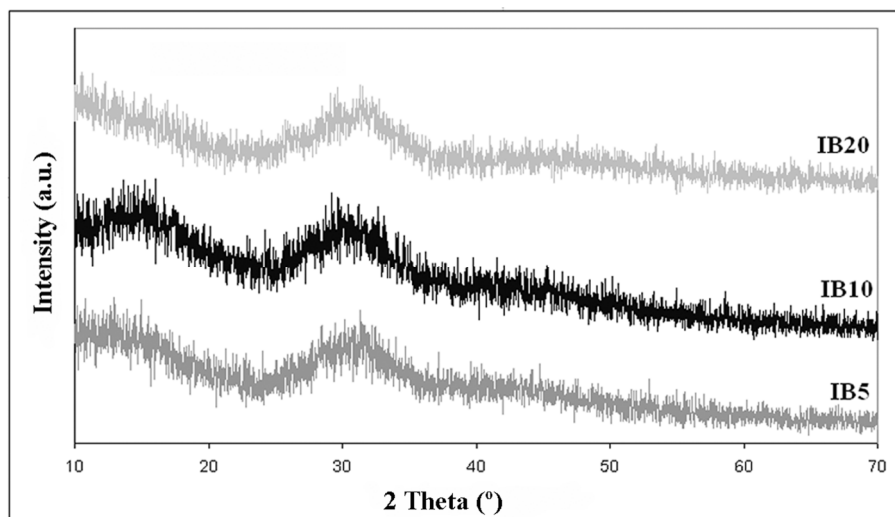


Figure 4: XRD patterns of composites after 21 days of soaking in PBS

Unlike other solutions that simulate the biological fluid, PBS does not have Ca in its composition and the absence of this element can delay the crystallization of the layer formed on the surface cement. In solutions where the Ca is present such as SBF (Simulated Body Fluid), there is usually a faster crystallization of calcium phosphate on the soaked materials surface. The PBS solution was chosen because it is a buffer solution, isotonic and non-toxic, which can give valuable preliminary indications on bioactivity.

Preceding work by these authors [40] has shown that PMMA-co-EHA composite filled with 50 wt.% of a silicate glass (the same glass used in this work) exhibited a fast formation of apatite in SBF, indicating that the composite became potentially bioactive and seems to be a suitable material for bone repair.

In order to confirm the crystallization of the layer formed on the surface cement a new immersion test using SBF was conducted. SEM images of composites and EDS spectra after immersion in SBF are presented in Figure 6. In contrast with PBS, the formed precipitates exhibited needle-like “cauliflower” morphology typical of apatite formation [8]. The composition of this layer was assessed by EDS. Before immersion, the presence of all elements that constitute the glass - Mg, Si, Ca and P - was detected (EDS spectrum not shown). After 14 days of immersion Ca and P were mainly identified, and the other elements like Mg, Cl and Na were residually present on the surface. The calculated Ca/P ratios for the several precipitates were 1.68 for IB5, 1.70 for IB10 and 1.65 for IB20 (Ca/P ratio for hydroxyapatite is 1.67)

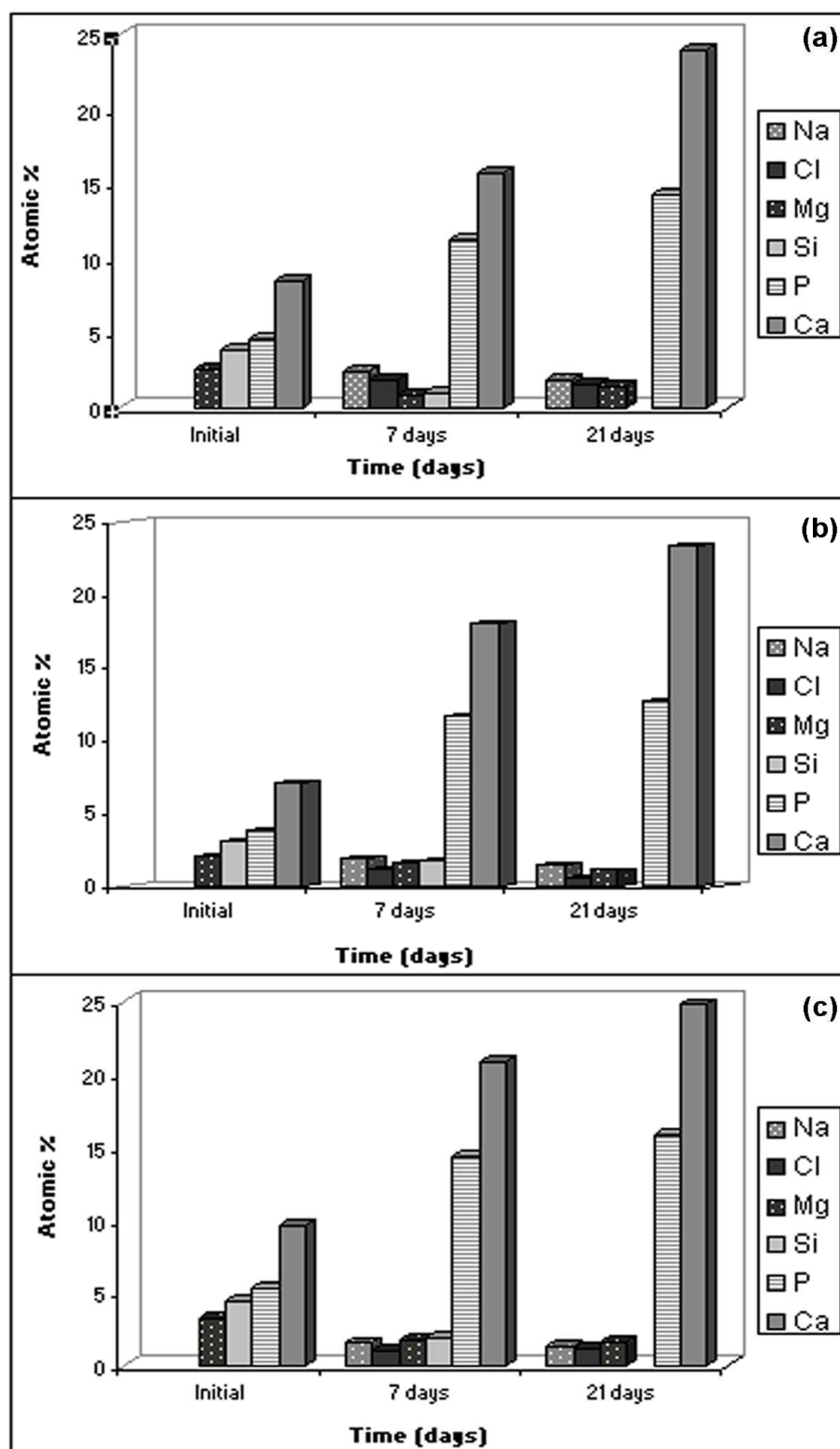


Figure 5: EDS profiles of the surface (a) IB5, (b) IB10 and (c) IB20. For each measurement a standard deviation of approximately 0.2 was determined.

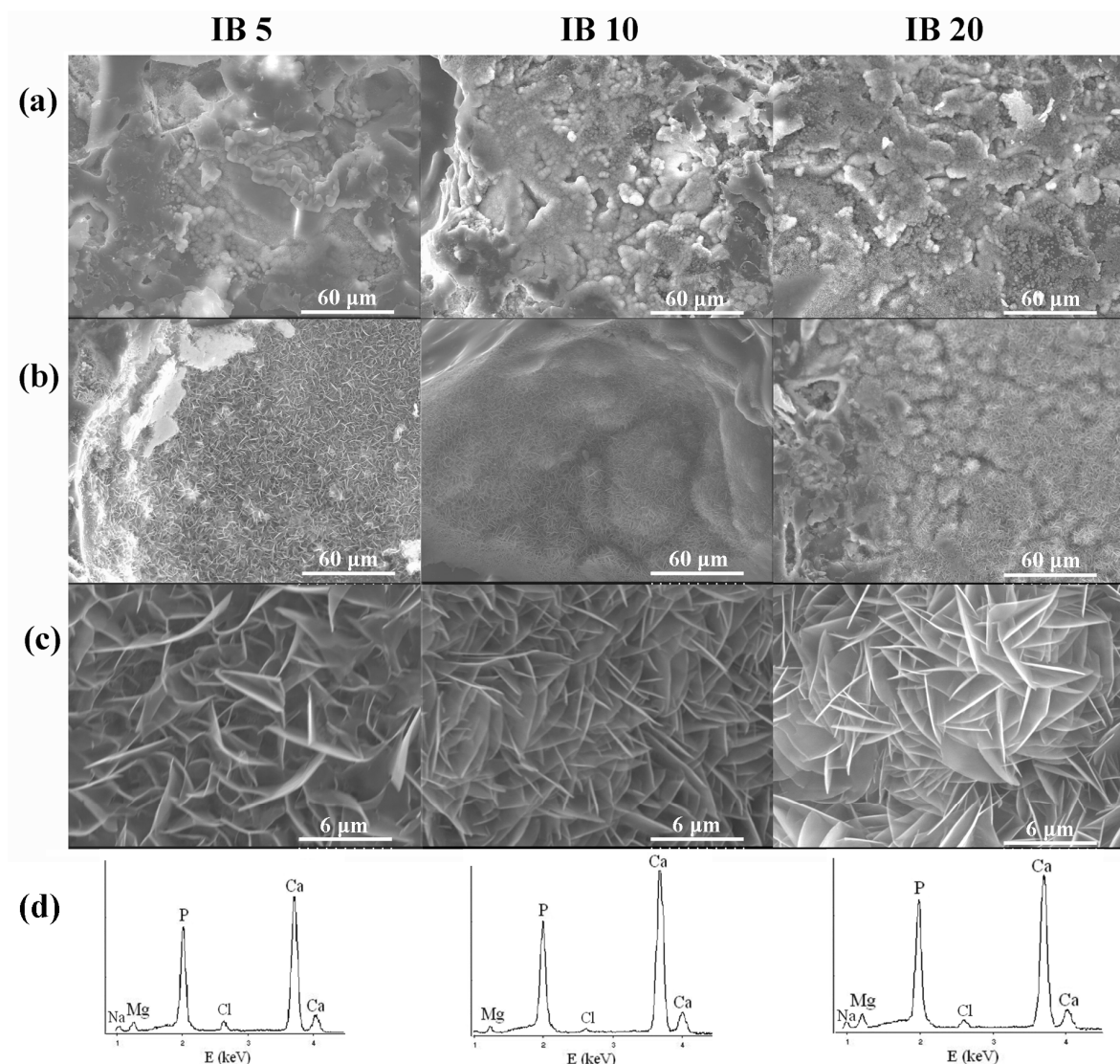


Figure 6: SEM micrographs of the cements surface (a) after 3 days, (b, c) after 14 days in SBF; (d) EDS spectra.

Analysis of the fluid by ICP (Figure 7) show that the release of Ca and P occurs up to 3 days of immersion, indicating dissolution of the glass, followed by its consumption which is associated to the formation of a calcium phosphate layer on the surface of the composite. The in vitro bioactivity of the fabricated cements was thus confirmed by these results. The morphology of the formed calcium phosphates is quite equivalent probably because the same percentage of bioactive glass was used in all formulations.

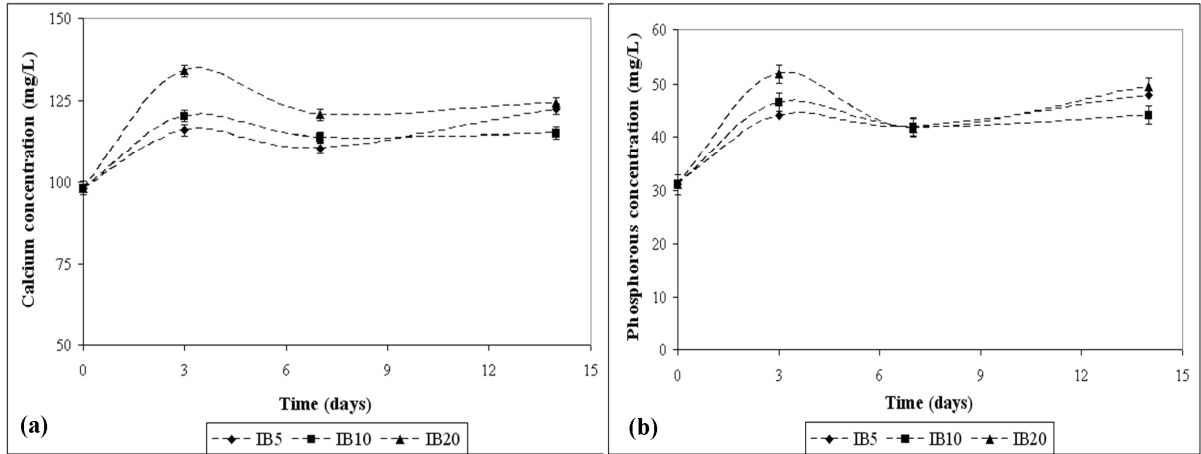


Figure 7: Variation in ionic concentrations of Ca and P in SBF. Dashed lines are to guide the eye.

Ibuprofen Release

The release of drug from an acrylic bone cement is a complex process in which it is expected a gradual liberation over time. The release curve of each ibuprofen concentration from the studied cements is shown in Figure 8.

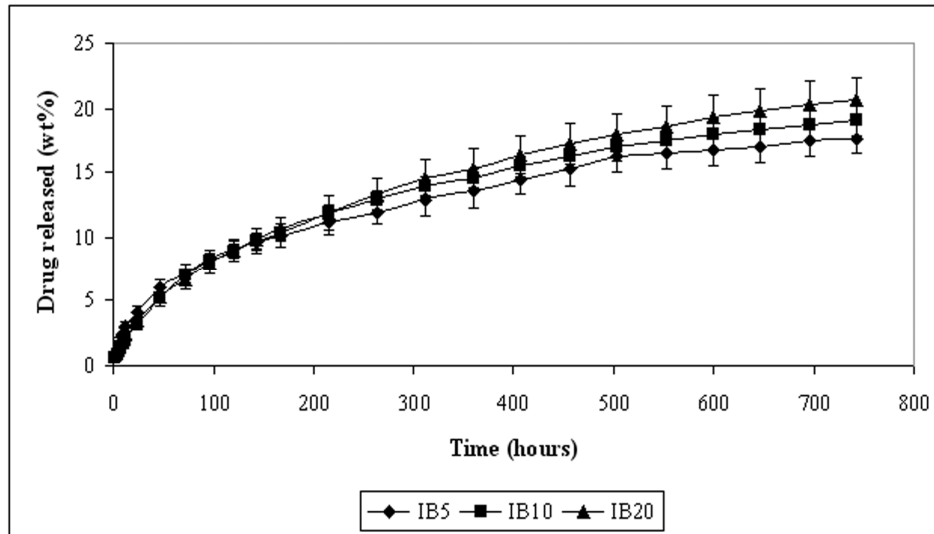


Figure 8: Ibuprofen release curves of the studied cements.

During 30 days test the cements released about 20% of the total incorporated ibuprofen. The highest percentage of drug released from the IB20, IB10 and IB5 was 20.6%, 19.1% and 17.6% respectively. During the first week of testing the proportion of drug released was not significantly different for the various studied amounts of ibuprofen. The delivery profiles showed that up to 7 days (168 h) 10% of ibuprofen content was released (half of the total amount of ibuprofen liberated) and between 7 and 30 days (168 to 720 h) the delivery rate is much lower, i.e. only about

10% was lost until the end of the test. Similar performances were reported in previous works, where it is suggested that the release was very incomplete because most of the drug beads were encapsulated by the hydrophobic PMMA matrix [41, 42].

The study of the release mechanism of ibuprofen was based on Equation 2. The analysis of the release curves can indicate the mechanism of drug liberation following the power-law model where n represents the release exponent. According to Cox et al, Fickian diffusional ($n \leq 0.45$) and Case II ($n \geq 0.89$) are the limits of this process only valid for samples of cylindrical shape like tablets [20]. Other values for n , between 0.45 and 0.89, indicate non-Fickian (anomalous) transport kinetics and can be described as a superposition of both phenomena.

In the present study the drug release mechanism from the bioactive cement follows an anomalous behaviour during the first week, with n varying from, approximately, 0.58 to 0.68 (Figure 9a). This behaviour may be due to the fact that only ibuprofen particles located in the superficial layers of the cement are accessible to the surrounding medium, leading to a competition between the dissolution of drug adsorbed to the material surface and the diffusion of drug close to the surface.

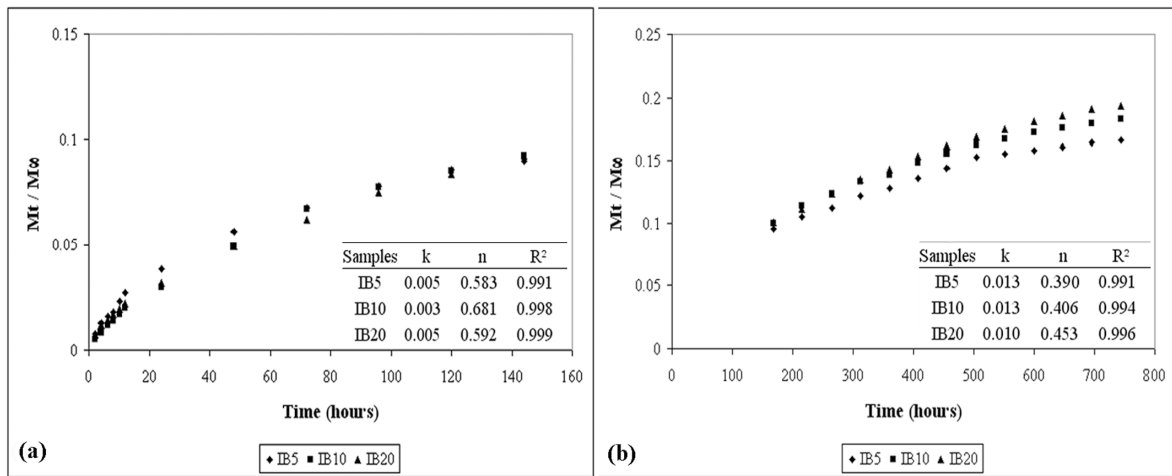


Figure 9: Release curve analysis and parameters according to power law (a) up to 7 days, (b) between 7 and 30 days.

For the time interval 7-30 days (Figure 9b), the release exponent indicates that the diffusion becomes the predominant mechanism (n varying from about 0.39 to 0.45). In this case the release occurs by the usual molecular diffusion of the drug due to a chemical potential gradient. The extremely low rate of transportation of the molecules is probably performed by bulk diffusion [11, 43].

The obtained results indicate that approximately 80% of ibuprofen was retained in the cement showing a very slow release of the drug. It is believed that the continuation of the slow liberation

along time is not harmful in vivo. Surface commercial bone cements loaded with antibiotics like gentamicin demonstrate similar release behaviour. In vitro experiments have shown that most of the antibiotics may be retained within the PMMA matrix; sometimes as much as 90% of the initial load [44-46] .

The drug release process may be ruled by the penetration of dissolution fluid into the polymer matrix, the solubility of the drug in solution and the possible physical or chemical interactions with the surface of the delivery device [11, 47]. Considering the cement after the MMA polymerization the drug particles are not free but embedded and covered by the PMMA matrix, which also encloses the bioactive glass and the PMMA beads initially added forming part of it as a homogeneous mixture. Hence the presented behaviour can be related with the low solubility of ibuprofen in aqueous media and the hydrophobicity of the developed formulations, which interfere in the contact with the dissolution fluid.

It is very important to try to predict whether the amount of ibuprofen released from the investigated cements has the suitable effect to potentiate the required therapeutic action in a real situation. As previously described the IB released from the cements was measured by UV spectroscopy and its corresponding concentration calculated from the calibration curve. Based on these results some calculations on the amounts of released drug from the different cement formulations were made and shown in Table 2, representing an exercise to predict the performance of the developed material.

When considering the drug release during the first week, period in which the inflammation is more critical, the loss of 10% of drug in a volume of 10 mL corresponds to a daily liberation of 0.063, 0.126 and 0.251 mg/mL of ibuprofen from respectively, samples IB5, IB10 and IB20. Since the therapeutic concentration of ibuprofen in human plasma is usually in the range 20 to 30 mg/L or 0.02 – 0.03 mg/mL [24] it is concluded that this value was reached by all formulations studied. Taking into consideration that the typical time action of an anti-inflammatory drug during one day, is 4 - 6 hours [23], then a minimum per day of 4 doses (0.08 to 0.12 mg/mL) or 6 doses (0.12 to 0.18 mg/mL) would be necessary to blunt the inflammation. Regarding our results this indicates that only IB5 would be ineffective in suppressing the inflammatory process and the IB10 and IB20 cements clearly achieved the therapeutic concentration required to control inflammation during the first week. It is believed that the high concentration reached by IB20 is not harmful since the drug is released over time. Moreover it was reported that in a symptom-free adult a level of 704 mg/L is tolerated [48, 49].

Table 2: Drug release of cements, based on UV spectroscopy measurements

Samples	Drug (mg)	First week (mg/mL)		Next week (mg/mL)	
		Total	Daily	Total	Daily
IB5	44	0.44 ± 0.02	0.063	0.128 ± 0.03	0.018
IB10	88	0.88 ± 0.02	0.126	0.322 ± 0.03	0.046
IB20	176	1.76 ± 0.05	0.251	0.685 ± 0.05	0.098

Concerning the second week the results indicate that the delivery rate is much lower and only 4-5% of ibuprofen is released. Thus, the compositions IB5 and IB10 showed insufficient daily dose of release for the treatment of inflammation. Only IB20 retained the drug liberation necessary to blunt the inflammatory process. This comparative study clearly evidenced that the performance of the cements with 10% and 20% of ibuprofen is quite satisfactory for their application in the control of inflammatory situations in bone surgery.

The extensive in vitro characterization of the developed materials here presented and the interesting capabilities found, encouraged the investigation of additional parameters such as mechanical and biological properties, a work that is being carried out in order to characterize the overall performance of the cement.

CONCLUSIONS

Bioactive bone cements with different contents of ibuprofen were synthesized. The characteristics of the curing process of the bioactive bone cements were improved with the increasing amount of ibuprofen leading to a reduction of the peak temperature and an extension of the setting time. A slight increase in the percentage of residual monomer in the cement was verified with the addition of drug. It was also shown that the presence of ibuprofen does not impede the in vitro bioactivity of the drug-containing composites. The growth of spherical calcium phosphate aggregates was observed on all composite surfaces.

The composites loose about 20% of the total incorporated ibuprofen during 30 days test. Release of drug is controlled by two different mechanisms: for the initial times the drug liberation is predominantly a surface phenomenon and for the longer times the ibuprofen release process is mainly controlled by diffusion. The cement formulation IB5 showed insufficient daily dose, i.e. lower than the required therapeutic concentration. The IB10 and IB20 cements achieved the therapeutic effect sufficient for the treatment of inflammation for one week and two weeks, respectively. The results evidenced that the performance of the cements with 10% and 20% of ibuprofen is quite appropriate to blunt the inflammatory process associated to orthopedic surgery.

Thus, the developed cements appear as interesting alternatives to traditional bone cements, allowing the surgeon to choose the most suitable formulation for each specific situation and patient.

Acknowledgements

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CHAPTER 5

ACRYLIC FORMULATIONS CONTAINING BIOACTIVE AND BIODEGRADABLE FILLERS TO BE USED AS BONE CEMENTS: PROPERTIES AND BIOCOMPATIBILITY ASSESSMENT

ABSTRACT

The solid phase of bioactive self-curing acrylic cements was modified by different biodegradable fillers such as poly(3-hydroxybutyrate) (PHB) and its copolymer with hydroxyvalerate (PHBV). The addition of the biodegradable fillers made the cement partially degradable, which is important to allow new bone replacement and ingrowth. The thermal analysis, crystallinity, curing parameters, mechanical properties, degradation and cellular tests were studied in order to characterize the cement performance. Within this context it was verified that the incorporation of the PHBV polymer made the cement more resistant, reaching values in the range reported for typical PMMA bone cements. The results also showed that the cement filled with PHBV took up more water than the cement with PHB after 60 days, for all studied formulations. Regarding the biocompatibility assessment, the inclusion of the PHBV greatly improved the biological response in both cements filled with the silicate or the borate glass, compared to the inclusion of the PHB. The importance of this approach resides on the combination of the properties of the cements components and the possibility of allowing bone regeneration, improving the interfaces with both the prosthesis and the bone, and leading to a material with suitable performance for application as bone cement.

This chapter is based on the following publication:

Lopes PP, Garcia MP, Fernandes MH, Fernandes MHV. Acrylic formulations containing bioactive and biodegradable fillers to be used as bone cements: properties and biocompatibility assessment. Biomedical Materials, submitted.

INTRODUCTION

The basic component of the acrylic bone cement is methyl methacrylate (MMA) which can polymerize to form poly(methyl methacrylate) (PMMA) [1]. Thus the cement itself is a typical bioinert material, that is, it does not resorb or allow bone replacement, being encapsulated by fibrous tissue [2]. The formation of fibrous tissue can be caused by the toxicity of the released monomer and the heat production due to polymerization, being a significant factor in the instability and movement at the bone-cement-prosthesis interfaces considered the weak-link-zones [3]. These micromovements can accelerate aseptic loosening, causing a failure in the cemented total hip arthroplasties [4].

In this context, the development of new formulations namely bioactive bone cements is highly desirable, since they can promote bone growth and the formation of a strong chemical bond between the implant and bone tissue [5, 6]. The incorporation of a bioactive component in acrylic bone cement formulations seems to be the main route to improve the interfacial strength of cement to the bone [7]. However, in most cases a significant part of the bioactive particles is covered with PMMA matrix and the contact with physiological solution is absent. Only the particles at the cement surface are accessible and react with the surrounding fluid, thus restricting the formation of calcium phosphate at the surface [8].

Certainly a much stronger interaction should be attained if the bone was induced to grow also inside the cement. An interesting approach would be to combine bioactive and biodegradable fillers within the solid component of the bone cement formulations, which could simultaneously facilitate bone replacement, ingrowth and bonding. It is believed that bone could grow around, as well as into, the cement in the space left by the degraded material resulting in stronger fixation of the prosthesis within the bone cavity [9].

Regarding this new class of bioactive bone cements containing degradable polymeric constituents, examples in the literature are relatively scarce. They include cements based on corn starch/cellulose acetate blends (SCA) filled with hydroxyapatite (HA) [10] or Bioglass [8], cements composed of chitosan and natural bone powder (HA obtained from trabecular bone blocks of porcine spines) [11] or cements containing microspheres of chitosan/ β -TCP [12].

Polyhydroxyalkanoates (PHAs) are natural polymers produced by many bacteria as a mean to store carbon and energy. They are known as biodegradable materials and exhibit a range of properties that may permit their use as biomaterials [13]. The most studied member of this family is poly(3-hydroxybutyrate) (PHB) which was discovered in 1920. PHB is relatively brittle and stiff, however its copolymer with hydroxyvalerate (PHBV) exhibits a less stiff behaviour and is tougher [14, 15]. These polymers slowly degrade *in vivo* by the enzymes present in blood, through

hydrolysis, and their degradation products are a common metabolite in human body, hence they are not toxic to the cells [13]. Another interesting property is its piezoelectricity which makes these polymers potential candidates for orthopedic applications, since electrical stimulation is known to promote bone growth and healing [15].

In the study presented herein, a new acrylic bone cement combining a biodegradable polymer (PHB or PHBV) and a bioactive glass filler (silicate-based glass or borate-based glass) is proposed. Experiments to measure mechanical properties, curing parameters and residual monomer were carried out as well as degradation and cytocompatibility studies. These results suggested that the new formulations may contribute to overcome some of the known drawbacks of acrylic bone cements which ultimately contributes to lower the incidence of implant aseptic loosening in cemented total joint replacements.

MATERIALS AND METHODS

Materials

Methyl metacrylate (MMA monomer), PMMA beads (polymer), 4,4-bis(dimethylamino) benzydrol (activator), poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV, PHV content 12 wt%) were acquired from Aldrich. PMMA beads, with a molecular weight of 120,000, were milled in a rotor mill (Retsch ZM 200) to a mean particle size of 100 μm . PHB (particle size of less than 50 μm) and PHBV, with the chemical structure depicted in Figure 1, are commonly produced via a controlled fermentation process using microorganisms.

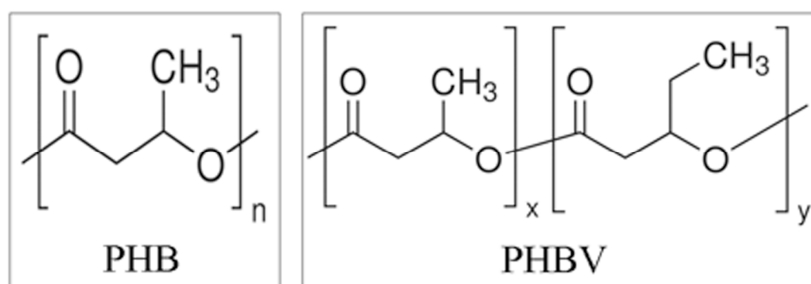


Figure 1: Chemical structure of used biodegradable polymers.

Commercial pellets of PHBV were frozen with liquid air and later milled in a rotor mill equipped with stainless steel knives to a powder with mean particle size of about 200 μm . PHB, Benzoyl peroxide (BPO, Merck) initiator and activator were used as received for the polymerization reaction. Two different glass compositions were used in this work. A silicate glass composed of (mol%) 24.5% SiO₂, 38% CaO, 12.7% P₂O₅, 24.8% MgO and a borate glass, which consists of a similar composition where SiO₂ was entirely replaced by B₂O₃. The glasses were

prepared through the classic melt-quenching method following a procedure described in Lopes PP [16, 17].

Cements preparation

New formulations of bone cements were prepared from a solid and a liquid phase by free radical polymerization. The solid phase was constituted by commercial PMMA, bioactive glass, biodegradable polymer and BPO. The activator of reduced toxicity was dissolved in the MMA monomer, resulting in the liquid phase.

All formulations contained a fixed percentage of 40 wt% of glass, referring to the weight of the solid component. Two bioactive glass compositions were used, a silicate glass and borate glass. The preparation of specimens for subsequent tests was carried out following the traditional method. Both phases were manually mixed with a glass bar until the mixture became dough with a high viscosity. Then the dough was placed into a poly(tetrafluoroethylene) (PTFE) mould and cured at room temperature. After 60 min, the samples were removed from the mould. The formulations were prepared by varying the composition of the solid phase, replacing 10 and 20 wt.% of the PMMA by the biodegradable polymer (PHB or PHBV). The compositions of all cements developed are presented in Table 1. The solid:liquid ratio employed was 1.75:1, with the initiator/activator in a molar ratio of 2.6.

Table 1: Chemical composition of all cements developed (wt%).

Samples	Solid Phase				Liquid Phase	
	PMMA	Bioactive Glass		Biodegradable Polymer		MMA
		GSi	GB	PHB	PHBV	
PMMA	63.6					36.4
PHB10Si	32.2	25		6.4		36.4
PHB20Si	25.9	25		12.7		36.4
PHB10B	32.2		25	6.4		36.4
PHB20B	25.9		25	12.7		36.4
PHBV10Si	32.2	25			6.4	36.4
PHBV20Si	25.9	25			12.7	36.4
PHBV10B	32.2		25		6.4	36.4
PHBV20B	25.9		25		12.7	36.4

Thermal behaviour and crystallization

To observe the effect of biodegradable polymer incorporation on the thermal properties of the cements, differential scanning calorimetry (DSC) experiments were performed (Shimadzu DSC-50). The experiments were carried out at a heating rate of 10 °C/min from room temperature up to 200 °C under constant nitrogen flow. After that the same samples were cooled to room temperature at 10 °C/min). The glass transition temperature (T_g) was taken as the midpoint of the first endothermic depression in the DSC plot. The variation in the degree of crystallinity (X_c) of the cements as a function of biodegradable polymer and their content in the composite was estimated using the transition enthalpies obtained from the DSC thermograms according to the equation:

$$X_c = \frac{\Delta H_m}{\Delta H_m^0 w} 100 \quad (1)$$

where ΔH_m and ΔH_m^0 are, respectively, the enthalpy of melting of the sample and the enthalpy of melting of fully crystalline PHB (146 J/g) or PHBV (109 J/g) and w is the weight fraction of the biodegradable polymer [18, 19]. ΔH_m is calculated by integrating the peak corresponding to the given transition.

X-ray diffraction (XRD) was also performed (Rigaku Geigerflex Dmax-C X-ray diffractometer equipped with a $\text{CuK}\alpha$ monochromatic radiation source) revealing information about the crystal structure of the cements.

Setting parameters

The temperature changes, which occurred at the setting reaction, were measured in a cylindrical mould at room temperature. Materials under testing were mixed in a small PTFE beaker for 1 min. The exothermic polymerization temperature was measured using a digital thermometer inserted in the curing mass at approximately 3 mm from the bottom of the tube, and was recorded every 10 seconds. Time was measured with a chronometer from the onset of mixing the powder with the liquid. Setting time (t_{set}) was calculated as the time at which the temperature of the mass corresponded to the sum of the ambient temperature (T_{amb}) and the maximum temperature (T_{max}) divided by two.

$$t_{\text{set}} \rightarrow T = \left(\frac{T_{\text{max}} + T_{\text{amb}}}{2} \right) \quad (2)$$

The measurements were done in duplicate, recording the temperature–time profiles during the polymerization.

Residual monomer

Nuclear magnetic resonance (NMR) spectroscopy was used to quantify the residual monomer content. Three samples of each type were dissolved using deuterate chloroform as solvent and tetramethylsilane (TMS 1 vol.%) as the internal reference and the ¹H NMR spectrum recorded on a 300 Bruker Avance Spectrometer operating at 300 MHz at room temperature. The percentage of monomer moles present in the cured cement sample (%*Mr*) was calculated using the following expression:

$$Mr = \left(1.5 \times \frac{A_v}{A_m} \right) \times 100 \quad (3)$$

where *A_v* and *A_m* stand for the area of vinyl and methoxyl signals, respectively and 1.5 is a factor relating the number of protons in the methoxyl group (three) to those of the vinyl region (two) [20].

Mechanical behaviour

The mechanical behaviour of the cured materials was evaluated under three-point bending. Samples were prepared in the same way to nullify any influence of the preparation technique upon the mechanical properties. The bending tests were carried out at room temperature on a Bose/Electro Force 3400 testing machine. Six samples per composition were tested at a crosshead speed of 1 mm/min. The bending strength (σ_B) and modulus (E_B) were calculated using the standard formulae [21]. The E_B data were extracted from the initial linear portion of the load - displacement curve.

$$\sigma_B = \frac{3 \times F \times L}{2 \times b \times h^2} \quad (4)$$

$$E_B = \frac{L^3 \times \Delta F}{4 \times b \times h^3 \times \Delta y} \quad (5)$$

where *F* is the highest load of the load-displacement curve, *L* is the distance between end supports, *b* is the width, *h* is the height of the specimen, ΔF and Δy are the gradient of load and displacement, respectively, of the initial straight-line portion of the curve.

The obtained values were compared with the ones indicated by ISO 5833 standard (strength 50MPa and modulus 1.8 GPa) [22].

Water uptake, weight loss and surface evaluation

These parameters of the self-curing materials were evaluated under simulated physiological conditions. The specimens were soaked in Phosphate Buffered Saline (PBS) at pH 7.4 and maintained at the temperature of 37 °C. A constant specimen surface area to solution volume ratio of 0.1 cm⁻¹ was used. At appropriate times, 3, 7, 14, 21, 28 and 60 days, the samples were blotted

on filter paper to remove surface solution/water, and immediately weighed. Water uptake (WU) and weight loss (WL) were calculated using the following equations:

$$WU = \left(\frac{m_t - m_o}{m_o} \right) \times 100 \quad (6)$$

$$WL = \left(\frac{m_{f,t} - m_o}{m_o} \right) \times 100 \quad (7)$$

where m_t stands for the mass of the sample “wet” after a given immersion time t (days), $m_{f,t}$ is the final mass after drying the sample in the oven until constant weight after t days of immersion in PBS and m_o is the mass prior to immersion [10].

The surface of all the investigated cements was evaluated by SEM-EDS before and after degradation test at 28 days of immersion in PBS. The samples were carbon-sputtered before observation under a scanning electron microscope (Hitachi S4100 model).

Statistical analysis

One-way ANOVA and multiple comparisons by the Tukey all pairwise approach were performed to determine the statistical significance ($p < 0.05$) of the differences among the groups. The comparisons were made among groups with the same biodegradable polymer and glass filler.

Osteoblastic cytocompatibility

Human bone marrow cell cultures

Cytocompatibility studies were performed with human osteoblastic cell cultures. Human bone marrow, obtained from orthopaedic surgery procedures, after patient informed consent, was cultured in α -Minimal Essential Medium (α -MEM) containing 10% fetal bovine serum, 50 μ g/ml ascorbic acid, 100 IU/ml penicillin, 2.5 μ g/ml streptomycin and 2.5 μ g/ml fungizone, at 37 °C in a humidified atmosphere of 5% CO₂ in air. Primary cultures were maintained until near confluence (10–15 days) and, at this stage, adherent cells were enzymatically released (trypsin–EDTA solution). First-passage cells were seeded at 2×10^4 cell/cm² over the surface of the cements. Seeded cements were cultured for 21 days in the presence of 50 μ g/ml ascorbic acid, 10 mM β -glycerophosphate and 10 nM dexamethasone. Colonized material samples were evaluated throughout the incubation time for cell viability/proliferation, alkaline phosphatase (ALP) activity and were observed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM).

Cell viability/proliferation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess the cell viability/proliferation. At days 4, 7, 14 and 21, colonized materials were incubated with MTT, during the last 4 hours of the culture time tested. The samples were placed in a new plate, the formazan salts were dissolved with dimethylsulphoxide and the absorbance was measured at 492 nm in a ELISA reader (Synergy HT, Biotek). Three replicates were set up at each condition. Results were compared in terms of macroscopic surface area and expressed as Acm^{-2} .

Alkaline phosphatase activity

At days 14 and 21, the colonized materials were treated with 0.1% triton in water (to lyse the cell layer) and the cell lysates were evaluated for ALP activity and total protein content. ALP was assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, 30 min at 37 °C, and colorimetric determination of the product (p-nitrophenol) at $\lambda=405$ nm. Enzyme activity was normalized to total protein content (determined by Lowry method). Results are expressed in nanomoles of p-nitrophenol produced per min per μg of protein ($\text{nmol}\cdot\text{min}^{-1}/\mu\text{g}$ protein).

CLSM and SEM observation

For CLSM, at days 4 and 14, colonized materials were fixed (4% formaldehyde, methanol free, 15 min), permeabilized in 0.1% Triton (5 min, RT) and incubated in 10 mg/ml bovine serum albumin with 100 $\mu\text{g}/\text{ml}$ RNase (1 h, RT). F-actin filaments were stained using Alexa-Fluor-conjugated phalloidin (1:100, 1 h, RT) and nuclei were counterstained with 10 $\mu\text{g}/\text{mL}$ propidium iodide (10 min, RT). Fluorescent stained cultures were examined by CLSM (Leica TCP SP2 AOBS confocal microscope).

SEM was performed at days 7 and 21. Material samples were fixed (1.5% glutaraldehyde in 0.14M sodium cacodylate buffer, pH 7.3, 10 min), dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analysed in a JEOL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDS) microanalysis capability (voyager XRMA System, Noran Instruments).

Statistical analysis

Data presented in the cytocompatibility studies were obtained from three separate experiments using cell cultures from different donors. Analyses were performed with three replicates. Groups of data were evaluated using two-way analysis of variance (ANOVA). Statistical differences between control PMMA and the prepared cements were determined by Bonferroni's method. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Thermal behaviour and crystallization

The developed cements were characterized in terms of thermal behaviour and crystallinity. DSC curves are shown in Figure 2.

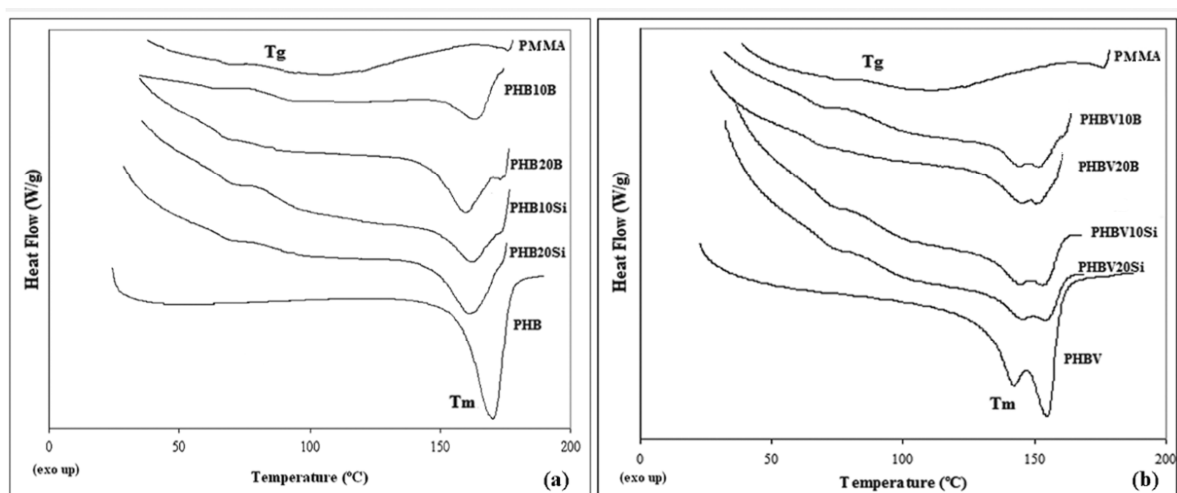


Figure 2: DSC thermograms of pure biodegradable polymers PHB and PHBV and studied cements.

A transition (first inflection point) was observed in all thermograms of the cements. This inflection was attributed to the glass transition temperature (T_g) of the amorphous PMMA matrix, as seen in the PMMA thermograms, which is around 86.7 °C (midpoint). The T_g is a physical parameter associated with the internal structural rearrangements produced by the softening of the material by heating, and represents a transition from a hard and rigid glassy state with a high modulus to a soft rubbery state with a low modulus [23]. The PMMA beads (polymer) used in the cement preparation have a T_g of the 105 °C (value obtained from Aldrich and confirmed by DSC). The lower T_g value for the PMMA matrix can be attributed to the lower molecular weight of the chains resulting from the MMA polymerization, and the presence of the residual monomer. The monomer probably acts as a plasticizer which increases the matrix flexibility and therefore produces a decrease in T_g [24]. The glass transition temperatures of acrylic bone cements can vary in the range 80 °C - 100 °C [25]. For the developed cements, there was a slight variation of T_g .

Filling the cements with PHB, which is a semicrystalline polymer, produces the appearance of endothermic peaks, corresponding to melting temperatures (T_m). It is observed that the T_m of PHB in cements slightly decreased (up to ~7 °C) when compared with the T_m of pure polymer (170.5 °C). This reflects the interaction of PMMA with the crystalline PHB, which leads to the T_m depression [26]. This behaviour may be explained by the low particle size and high surface area of

the PHB that can promote the incorporation of a higher amount of monomer. Thus it is believed that the presence of MMA can decrease the cohesion between the chains in the PHB crystals causing a reduction of the T_m .

For pure PHBV two melting temperatures are found and both are lower than that of PHB. The lower values may be related to a slight reduction in crystal size and lower degree of crystallinity of this polymer. For the melting of PHBV the main peak was detected around 155 °C and the second at approximately 143 °C. The latter is usually due to melting of crystals with different lamellar thickness and/or crystallization that occurs during the heating in the DSC [27]. For cements filled with PHBV the melting peaks became narrower and slightly shifted to higher temperatures, although the main peak was not considerably different.

DSC curves, obtained during the cooling stage (data not shown) indicated that the crystallization temperature was also lower for PHBV (109 °C) than that determined for PHB (120 °C). The crystallinity of PHB and PHBV phase can be calculated, according to the heat of fusion obtained from the interior areas of the melting peaks being illustrated in Figure 3. The degree of crystallinity of the pure PHB is higher than that of PHBV, due to the most irregular and random repeat unit arrangements in the copolymer which results in reduced ability of crystallization.

The results also showed that the crystallinity of PHB is much larger in pure polymer than in the cement, while for the PHBV the crystallinity seemed to be unchanged by the presence of PMMA. As mentioned before, this behaviour can be associated with the lower average particles size of PHB, around 50 μm , compared to the PHBV, 200 μm . A higher surface area as in PHB is responsible for a higher reaction between MMA monomer and PHB.

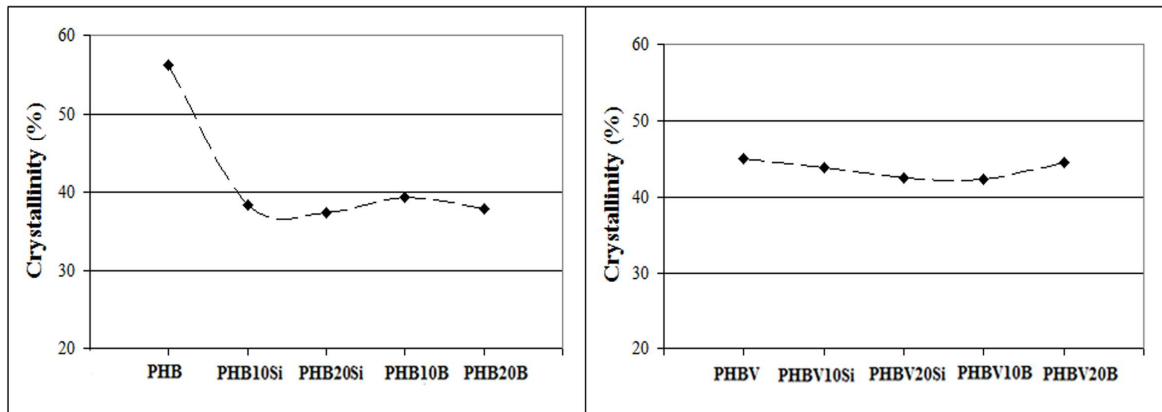


Figure 3: Variation in the degree of crystallinity of the PHB and PHBV in the cements.

Figure 4 shows the obtained XRD spectra for PHB, PHBV and developed cements. It can be seen that both PHB and PHBV are semicrystalline polymers, and exhibit peaks at almost the same locations, indicating that addition of PHV to PHB does not modify the crystal structure of PHB.

The main diffraction peaks appear around $2\theta = 13.6^\circ$, 17.0° , 22.4° , 25.6° , and 30.7° , which correspond to (020), (110), (111), (121), and (002) crystallographic planes, respectively [28].

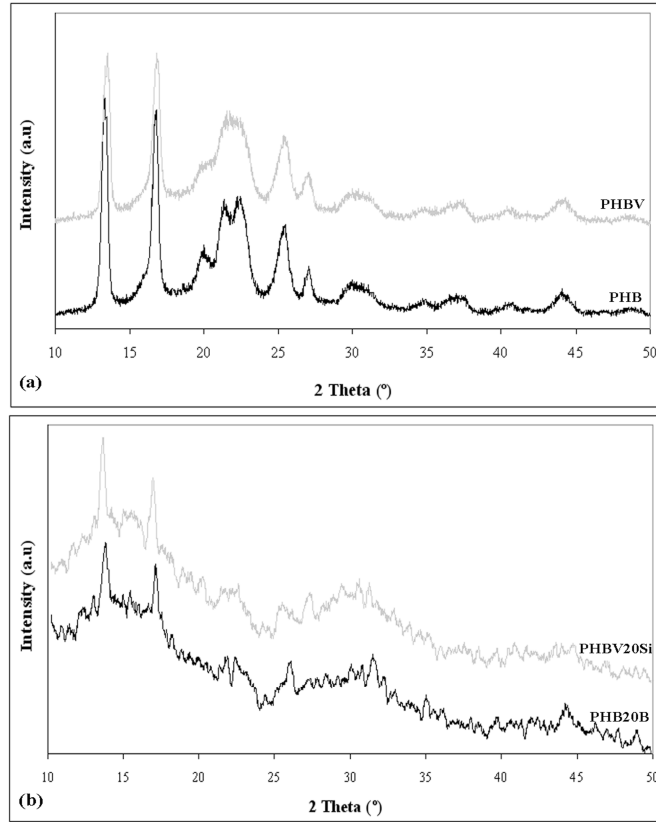


Figure 4: XRD patterns of (a) PHB and PHBV, (b) PHB20B and PHBV20Si.

It is known that, in the crystalline state, PHB adopts a regular helical conformation with space group $P2_12_12_1$. The unit cell is orthorhombic with dimensions $a = 5.76 \text{ \AA}$, $b = 13.20 \text{ \AA}$, and $c = 5.96 \text{ \AA}$ [29]. The PHBV polymers display the phenomenon of isodimorphism, i.e. Hydroxybutyrate (HB) and hydroxyvalerate (HV) units of the copolymer are incorporated in the same crystal lattice, given that the structures of both units and the crystal structures of the PHB and PHV are quite similar. Therefore, PHBV copolymers with less than 40 mol% HV units crystallize in the PHB crystalline lattice [19].

The XRD patterns for the developed cements (Fig. 4b) also exhibit diffraction peaks, characteristic of the biodegradable polymers. The introduction of crystalline polymers, such as PHB and PHBV, in the acrylic cement (an amorphous polymer where glassy particles are already embedded) gives rise to crystalline regions (spherulites) dispersed in an amorphous material. X-ray diffraction has additionally shown that the diffraction peaks of PHB were slightly shifted to higher 2θ angles indicating a slight change in the lattice parameters of the polymer which can be related

with the presence of MMA. It was also revealed a decrease in the intensity of these peaks, when compared with PHBV20Si, due to the reduction in the crystallinity of PHB.

Setting Parameters and Residual Monomer

The exothermic character of the polymerization reaction was evaluated by the setting parameters. Figure 5 shows the curing curve for each of the developed cements, as well as for PMMA reference, whereas Table 2 presents the values for the studied parameters.

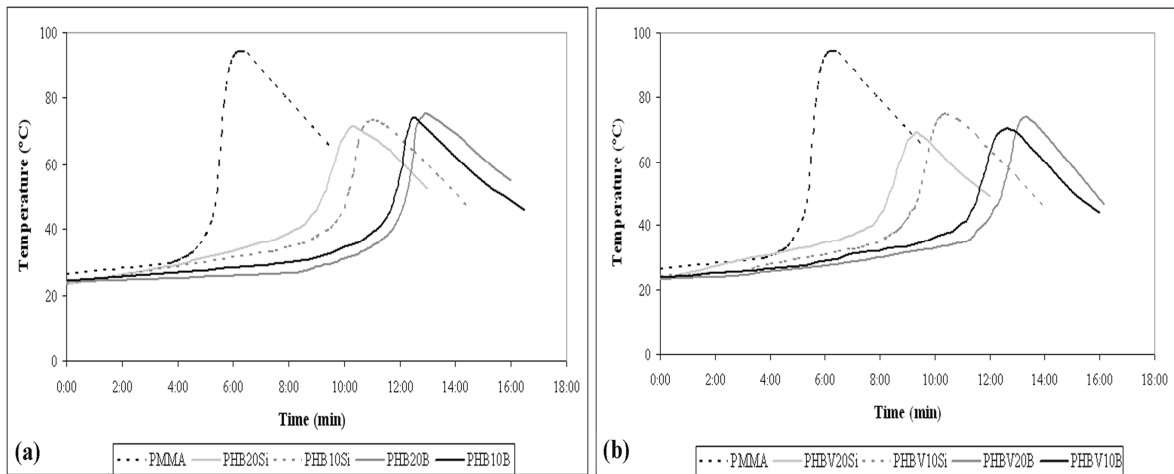


Figure 5: Curing curves of the developed cements filled with (a) PHB and (b) PHBV.

In terms of the maximum reached temperature, Figure 5 clearly shows that, when compared with the formulation based on PMMA, the addition of a biodegradable polymer and a bioactive glass, significantly decreases this parameter ($p < 0.05$). In fact for the cements modified with biodegradable and bioactive filler the peak of temperature achieved was less than 90 °C, which is the highest value accepted by the ISO 5833 standard for acrylic resin cements [22]. These results are indeed beneficial, since they indicate that with the developed cements the necrosis in the tissue surrounding the implant might be considerably minimized. The concentration of biodegradable filler did not promote greater changes in the peak temperature, except for PHBV10Si and PHBV20Si for which compared group means showed significant differences. In general and considering the different used biodegradable fillers, PHBV cements demonstrated less intense peak temperatures than the PHB cements and PMMA matrix.

The setting time values were significantly different for cements with several concentrations of biodegradable polymer, except between PHB10B and PHB20B. The cement filled with PHB and silicate glass reached a t_{set} higher than the cement filled with PHBV and the same glass. For the cement with borate glass the change of biodegradable polymer was not significant. The developed cements showed a large increase of the setting time which can be due to higher concentration of

fillers and the reduced amount of activator, providing a longer time for the preparation and application of the bone cement during surgical procedure. When the PMMA beads are substituted by filler the polymerization can be retarded, increasing the setting time [30]. Additionally, the formation of radicals is dependent on the concentrations of activator and initiator. Faster radical formation activates more monomers that act as nucleation sites for polymer chain growth [31]. Regarding the kinetics, decreasing the amount of activator decreases the rate of polymerization and, consequently, extends the setting process. The reduction of the maximum temperature is also expected since the heat of polymerization is released for an extended time [32, 33]. In literature it is reported that changing the initiator/activator ratio produces minor effects or conflicting results in polymerization temperature [31, 34]. In this work the reduction of activator seems to affect mainly the setting time.

The percentage of residual monomer in the different formulations ($M_r\%$) is summarized in Table 2. Comparing with the PMMA matrix, the addition of either PHB or PHBV produces an increase of the residual monomer present.

Table 2: Values of setting parameters and M_r for the investigated cements

Samples	T_{\max} (°C)	t_{set} (min)	M_r (%)
PMMA	97.3 ± 3	$5:30 \pm 0.18$	4.10
PHB10Si	73.4 ± 2	$10:05 \pm 0.15$	4.74
PHB20Si	71.6 ± 1	$9:05 \pm 0.18$	4.75
PHB10B	74.0 ± 2	$11:50 \pm 0.18$	5.40
PHB20B	75.3 ± 2	$12:15 \pm 0.14$	5.59
PHBV10Si	75.1 ± 1	$9:25 \pm 0.18$	4.54
PHBV20Si	68.2 ± 2	$8:15 \pm 0.18$	4.30
PHBV10B	70.3 ± 1	$11:45 \pm 0.21$	4.14
PHBV20B	73.3 ± 2	$12:40 \pm 0.18$	4.39

The cements filled with PHB led to larger amounts of residual monomer, that slightly increase with the increasing concentration of this biodegradable polymer. The cements filled with PHBV showed smaller values but still higher than for the PMMA matrix. The filler can act as an array of barriers, thus the overall probability of a monomer molecule to react with an initiator radical or a growing radical chain is decreased, resulting in a higher concentration of unreacted monomer. Another probable reason to justify this dissimilar behaviour can be the lower particle size of the PHB polymer. The superior surface area of the filler may immobilize more monomer on its surface

and therefore increase the non-reacted monomer that remains trapped in the cement matrix [30, 35]. These values were in the range reported in the literature [36, 37].

Mechanical Properties

The evaluation of mechanical properties is essential to determine the viability of self-curing acrylic formulations as potential systems for load-bearing applications. The bending test is a characterization technique to assess new cements, being a requirement of the standard, because in vivo loading invariably involves a combination of shear, tension, and compression forces [22, 38]. The representative nominal load – displacement bending curves for the studied cements are illustrated in Figure 6.

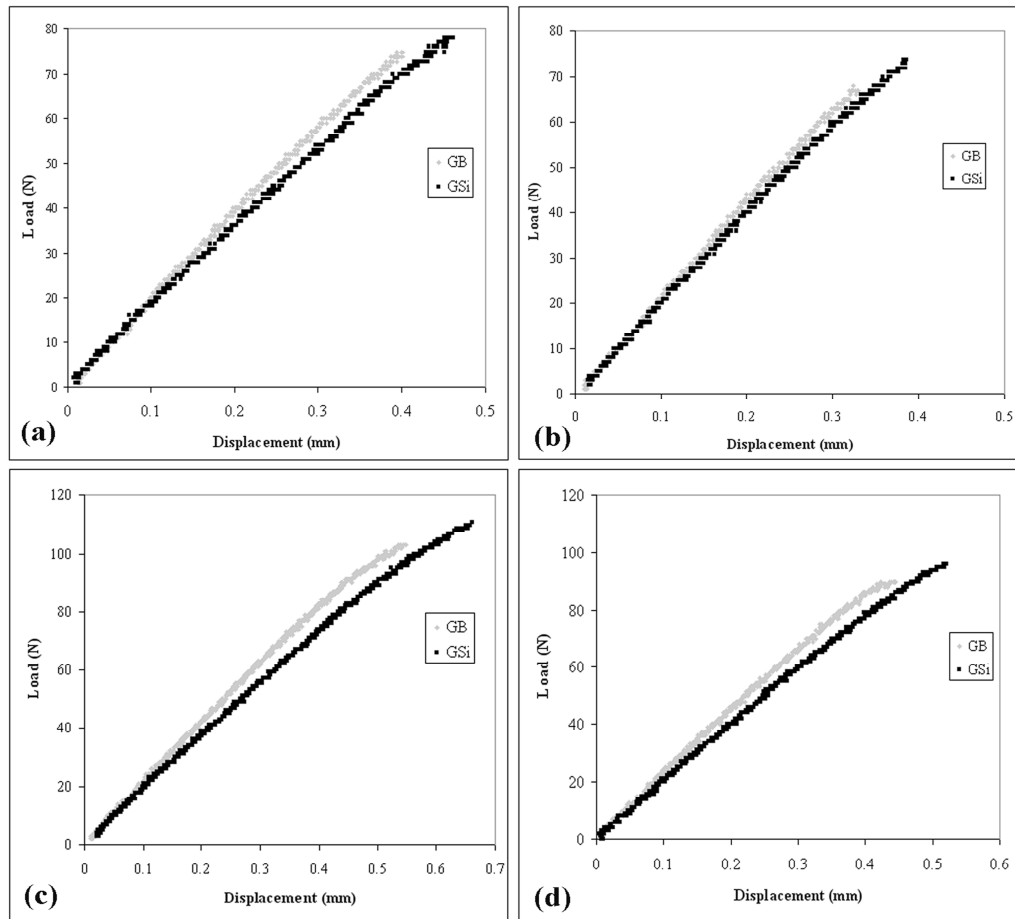


Figure 6: Load-displacement curves for the cements (a) PHB10, (b) PHB20, (c) PHBV10 and (d) PHBV20.

It is clear that the partial substitution of the PMMA beads by biodegradable particles retained the stiff and brittle behaviour of the material. A notable effect was verified when comparing the mechanical properties of the PHB-filled cements with those of the corresponding cement filled

with PHBV. Overall, the cement containing PHBV and silicate glass becomes more resistant since a greater load may be sustained by the specimen. The strength at maximum load and the elastic modulus for each composition are depicted in Figure 7.

One way ANOVA analysis indicated that the values obtained for the bending strength of the various cements were significantly different ($p < 0.05$). To explore the differences among the means, Tukey's multiple comparisons were performed. Considering the effect of addition of different biodegradable polymers, PHB and PHBV, (maintaining the same concentration and glass filler) all group differences were significant. However taking into account the concentration of biodegradable filler, 10 wt% and 20 wt%, (keeping the same polymer and glass filler) the result showed that there was no statistical difference, except between PHB10Si and PHB20Si. The elastic modulus was considered similar, it did not differ significantly, except between PHB10Si and PHBV10Si. In addition, the results also showed a slight increase in the bending strength for the cement filled with silicate glass and, considering the elastic modulus, the performance was slightly superior for the cement with borate glass. Nevertheless, as mentioned above, the statistical analysis resulted in not significant differences ($p > 0.05$).

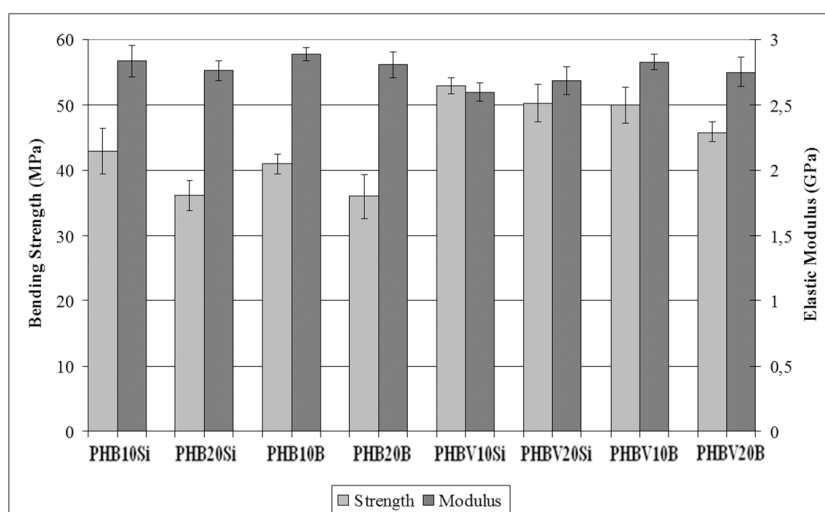


Figure 7: Bending properties of all the prepared formulations.

The observed behaviour seemed quite interesting, particularly in which concerns the pronounced effect of the different biodegradable polymers, rather than their concentration, in the mechanical properties of the modified cements. In terms of the influence of addition of PHB and PHBV, the incorporation of PHBV led to better bending strength but similar elastic modulus, all higher than 2.5 GPa.

It is known that PHB is a semicrystalline polymer with higher degree of crystallinity, being a relatively brittle and stiff material [39]. The reason for the brittleness is mainly attributed to the

presence of large crystals in the form of spherulites. The PHB is usually crystallized by slow cooling from the melt, forming thin lamellar crystals, which are organized in large banded spherulites [40]. The large-size spherulites and secondary crystallization promote interspherulitic cracking during storage of the polymer at room temperature, which is commonly known to impair the mechanical properties of the materials [41, 42]. So the bending strength of the cement is lower when PHB is incorporated, a behaviour that can be associated to the high viscosity of these samples during preparation which induces an inhomogeneous distribution and agglomeration of particles. These aggregates behave like preferential sites of stress concentration in the bone cement, thereby weakening it. In the present case we believe that, apart from the referred factors, the alteration verified in the thermal analysis of PHB, can also contribute negatively for the mechanical performance of the cements.

The random copolymer (PHBV) shows increased flexibility, since it generally produces a greater disorder within the crystalline region due to the ethyl side groups, thus the crystallinity is decreased, and the mechanical properties can be increased [43]. The reduced crystal growth of the PHBV can also be advantageous for the mechanical properties because smaller crystals are produced, which are more flexible than large crystals that are prone to brittle failure at the grain boundaries. The results evidence the high performance of the cements filled with PHBV, exhibiting mechanical properties that lie within the required range for bone cements use.

Degradation and surface analysis

Figure 8 shows the evolution of the water uptake (WU) and the loss of weight (WL) with time. The uppermost values of WU were obtained after 60 days of immersion in PBS. The WU values are influenced by the composition of the glass.

The cements filled with borate glass expressed a higher value of WU than the control (PMMA), while for the cements filled with silicate glass the WU data were lower/similar up to 28 days, but increasing for 60 days of immersion. The results for both types of cements indicated that the compositions containing 20% of biodegradable polymer demonstrated a superior WU capability compared to the one formulated with 10% of polymer during the same period. Regarding the different biodegradable fillers, the cement filled with PHBV took up more water than the PHB cement after 60 days, for all studied formulations.

The WL was also higher for the cement with 20% biodegradable polymer and borate glass, being that the PHBV20B showed the highest weight loss. Overall the weight loss of the cements reached a maximum value at 21 days of immersion, except for the PHB20B whose maximum WL was achieved at 7 days, and then decreased, indicating that the material should be affected by another process besides the degradation during immersion. An explanation for the reduction in WL

values can be related with the possibility of precipitation of a calcium phosphate layer on the material surface, that becomes evident after 21 days immersion [44]. The pH did not show substantial changes until the end of the test, ranging from 7.4 to 7.7.

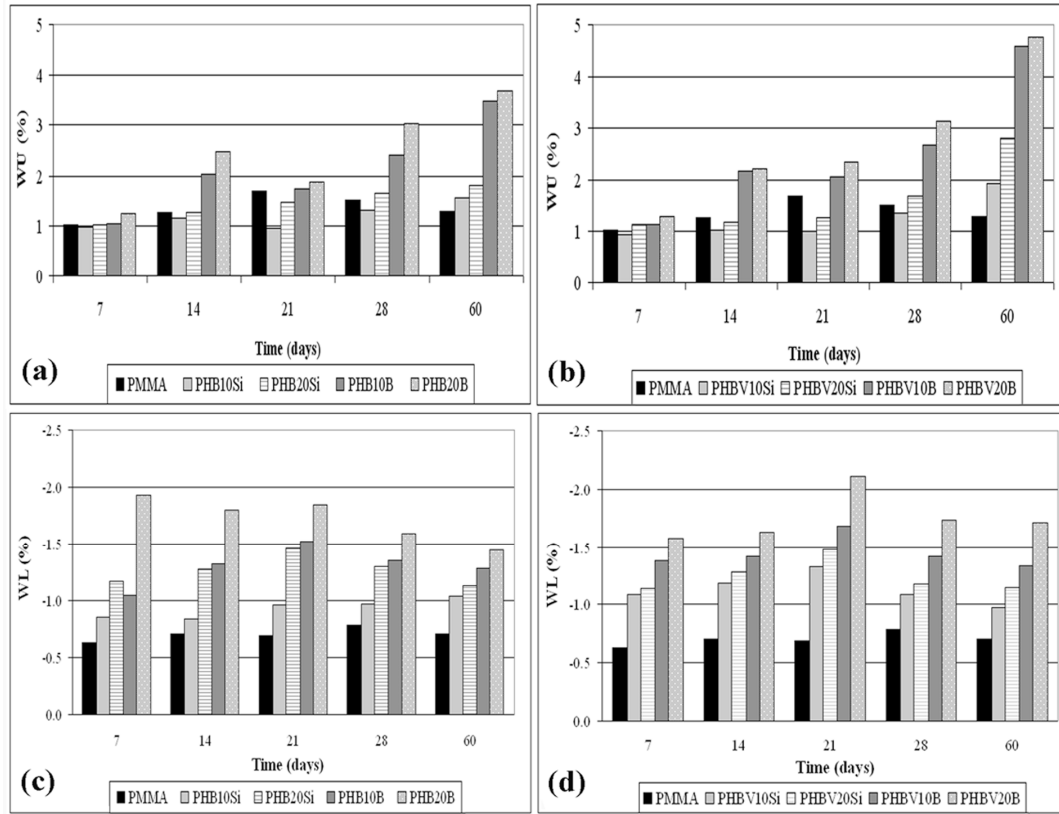


Figure 8: Evolution of water uptake (WU) and loss of weight (WL) for the cement filled with PHB (a, c) and cement filled with PHBV (b, d).

To verify the leaching process from the cements during soaking in PBS, scanning electron microscopy (SEM) micrographs were taken from all investigated formulations before and after PBS immersion for 28 days (at different magnifications). The surface of the cements, modified with PHB and PHBV, can be seen in Figure 9.

The micrographs revealed a quite similar behaviour for both cement fillers PHB and PHBV. Before immersion all the cements exhibited a PMMA matrix surrounded by the fillers (polymer and glass) and few pores. After 28 days in PBS the materials showed increasing porosity pattern, i.e. new cavities were formed at their surfaces. For PHB10Si and PHBV10Si these cavities are less evident, a result that is consistent with the degradation test where the same cements revealed a less weight loss. Concerning the cements filled with the borate glass it seems obvious that the porosity substantially increased, thus contributing for a higher loss of weight of these cements.

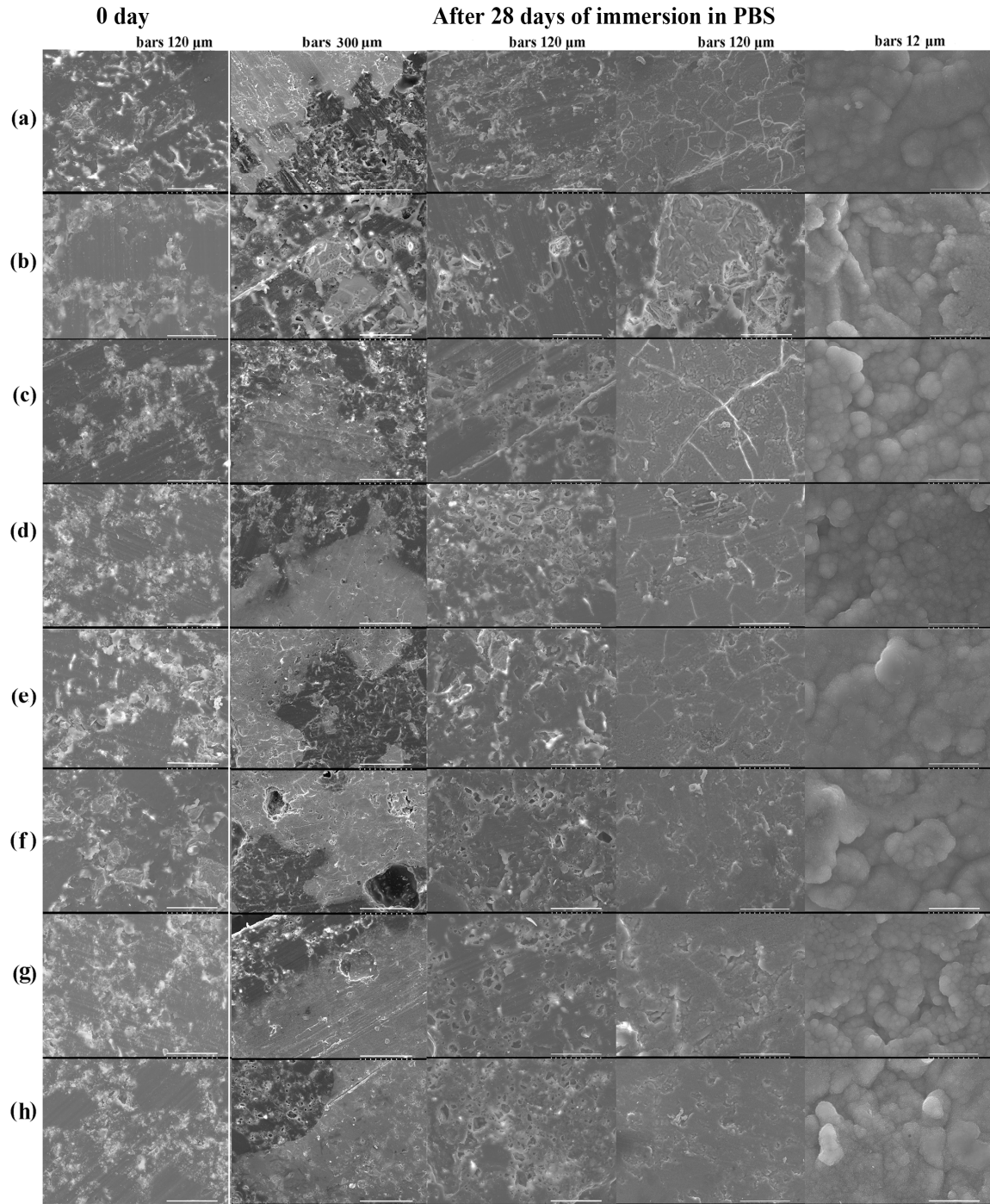


Figure 9: SEM micrographs of cements before and after 28 days of immersion in PBS (a) PHB10Si, (b) PHB20Si, (c) PHB10B and (d) PHB20B, (e) PHBV10Si, (f) PHBV20Si, (g) PHBV10B and (h) PHBV20B.

In all cements, after 28 days in PBS, it was also detected the presence of another phase covering a large surface of the samples. The precipitates, developed on the cement surface, showed a spherical morphology, small size and low crystallinity in higher magnification. It is known that PBS does not contain calcium in its composition and the absence of this element can delay the

crystallization of the formed precipitates [45]. These new layers were analyzed by EDS (data not shown). In terms of its composition, at least qualitatively, they were all similar. In all cements the developed precipitates were composed only by Ca and P, with residual amounts of Si (present in the silicate glass under the layer), Na and Cl (precipitated from the solution).

The presence of different biodegradable polymers seems not to influence the surface changes of the cements, at least for the investigated immersion time. The PHB and PHBV are known to degrade very slowly in an aqueous medium because of their degree of crystallinity and hydrophobic structure, and thus the superficial degradation of the cement could be mainly attributed to the dissolution of the bioactive glass which gave rise to the observed porosity and contributed to the formation of the calcium phosphate layers.

Osteoblastic cytocompatibility

Figure 10A shows the cell viability/proliferation (MTT assay) of PHB and PHBV cements seeded with human bone marrow cells, and cultured in conditions that favour osteoblastic differentiation [46]. At day 4, osteoblastic cells presented similar values on PMMA (control) and on the prepared cements. Cell growth increased with culture time in all materials, except in those filled with the borate glass and containing the polymer PHB. However, the various formulations presented significant differences regarding the cell growth rate and pattern.

PMMA showed a low growth rate during the culture time. The best performance was observed with the cements filled with silicate glass and containing the polymer PHBV. The highest values were achieved with the cements PHBV10Si and PHBV20Si, followed by the cements PHB10Si and PHB20Si. Regarding the cements filled with borate glass, the samples containing the polymer PHBV (PHBV10B and PHBV20B) presented relatively high cell growth, although lower than that observed with the cements filled with the silicate glass. The cements with the polymer PHB (PHB10B and PHB20B) exhibited the poorest cell response. Cell growth over PHB10B increased until days 7 – 14 and decreased in the last week, but cell behaviour was worse over PHB20B, with an impaired cell proliferation from day 7 onwards.

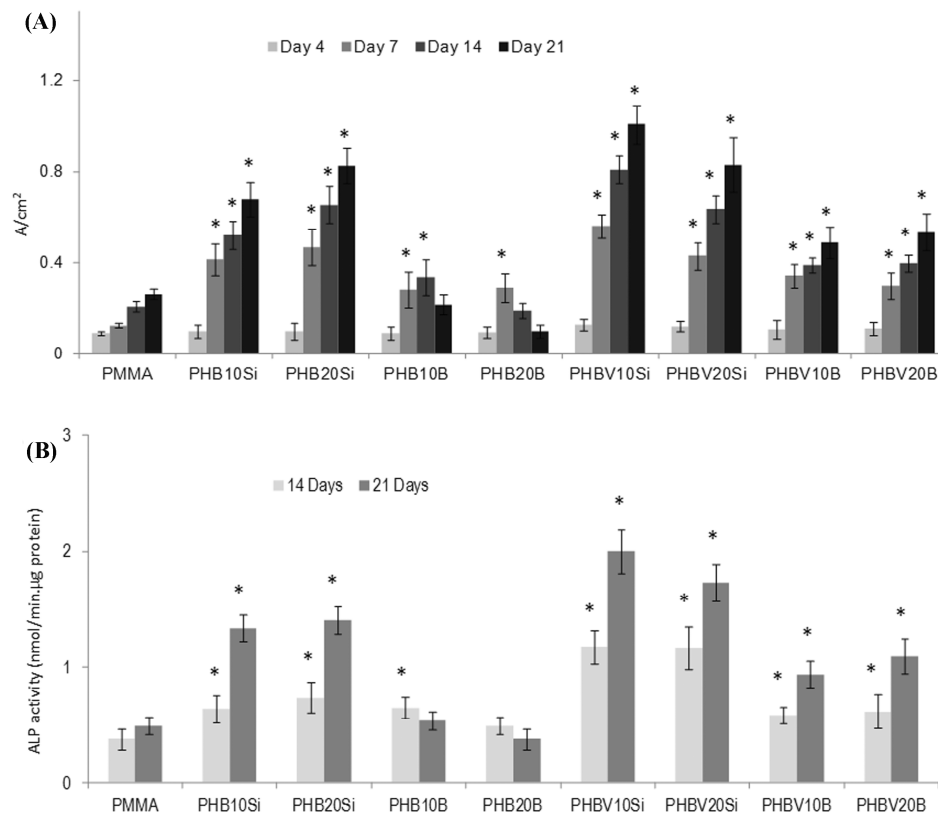


Figure 10: Cell viability/proliferation (a) and ALP activity (b) of human bone marrow osteoblastic cells seeded over the cements for 21 days. *Significantly different from PMMA.

A similar pattern was observed for ALP activity, measured at days 14 and 21, Figure 10B. PMMA presented low values and, except for PHB10B and PHB20B, enzyme activity increased from days 14 to 21, suggesting an osteoblastic differentiation pathway [47]. Also, the highest values were observed for the silicate cements and containing the polymer PHBV.

CLSM observation of colonized materials, at days 4 and 14, were in agreement with the results observed in the MTT assay, Figure 11. At day 4, cells were well attached and spread, displaying a flat configuration and a typical morphology (central spherical body with the cytoplasm extending away from the central area in all directions and adhering to the material surface), and cell-to-cell contact through cytoplasmic extensions. The cements showed a similar appearance, but a higher cell number appears to be observed in the cement PHBV10Si. At day 14, the cements containing the silicate glass and the PHBV polymer presented the most abundant cell layer, whereas the cements with the borate glass and the PHB polymer displayed the poor performance.

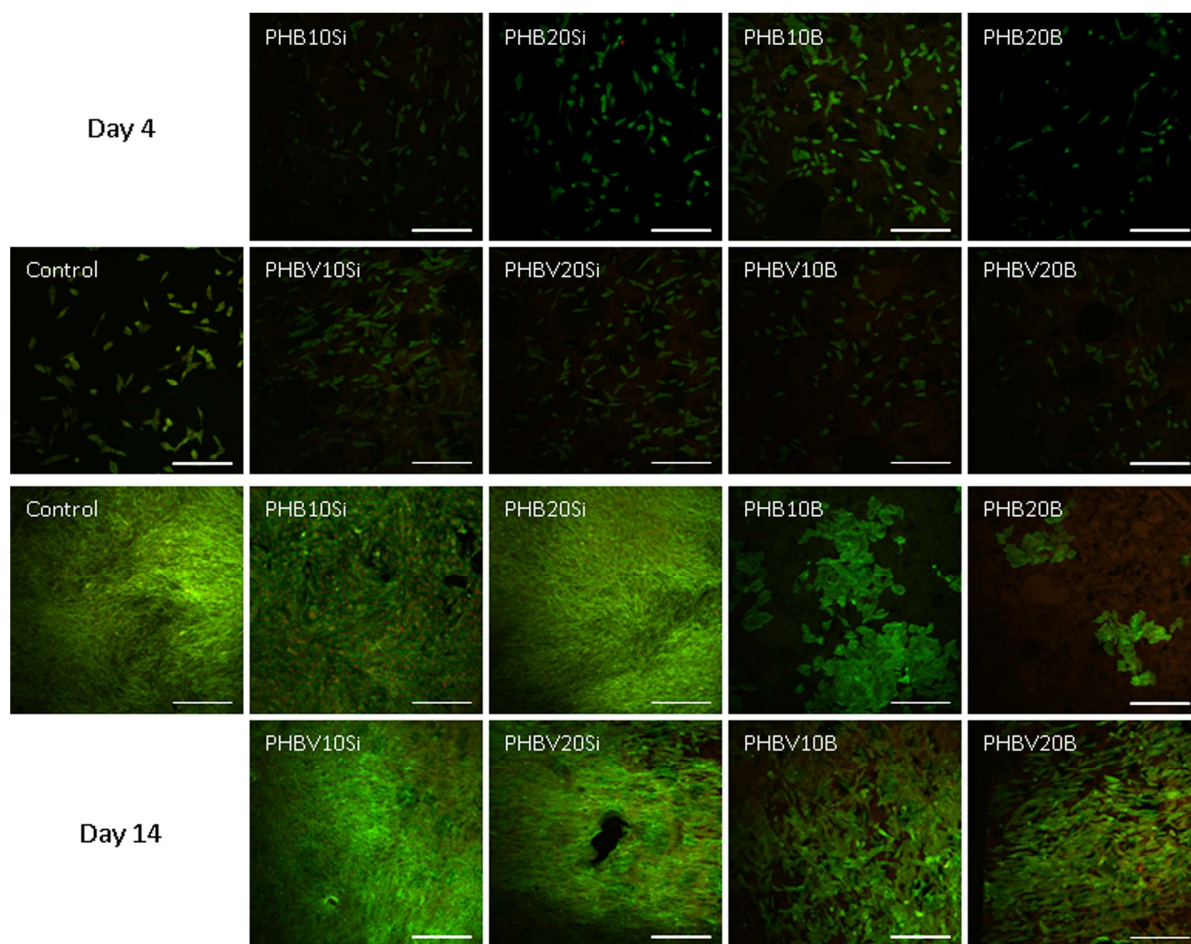


Figure 11: Representative CLSM images of human bone marrow osteoblastic cells seeded over the cements for 4 and 14 days; bar = 400 μ m.

SEM observation of the material samples also confirmed this behaviour. Figure 12 shows representative images of the cements cultured for 21 days. The cements PHBV10Si and PHBV20Si presented an organized cell layer with cell-to-cell contact that successfully adapted to the underlying surface. Comparatively, the samples PHB10Si and PHB20Si showed a similar appearance, although with a less abundant cell layer. The cements PHBV10B and PHBV20B also presented spread cells with an elongated morphology, some cell-to-cell contact, but areas of continuous growth were rarely seen. PHB10B and PHB20B exhibited few isolated cells scattered over the material surface.

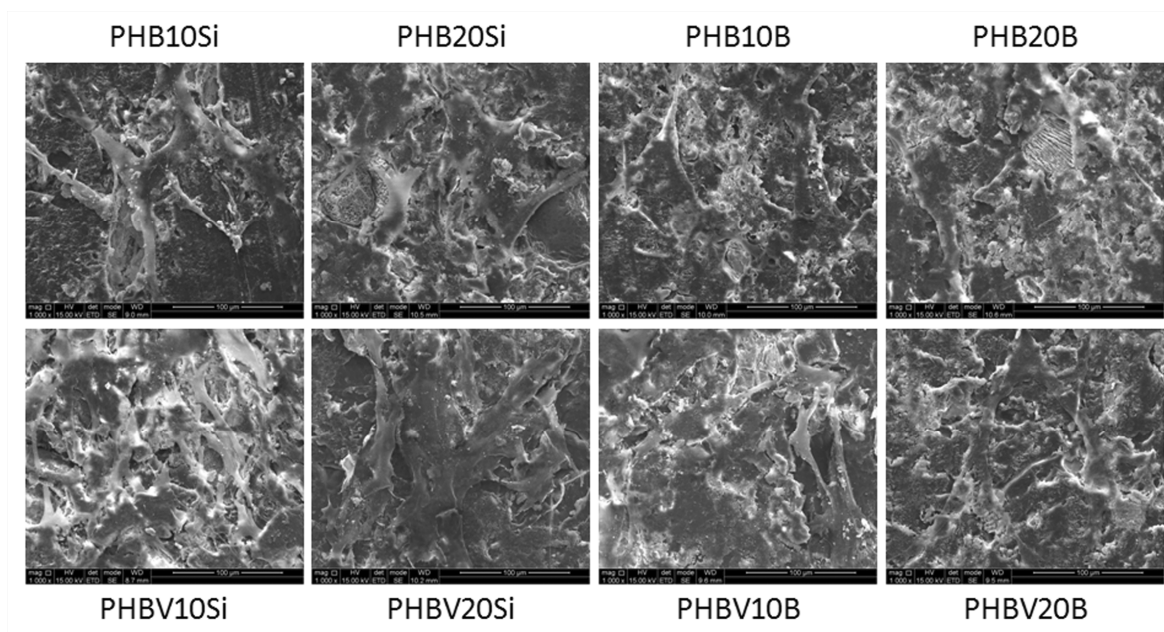


Figure 12: Representative SEM images of the cements cultured with human bone marrow osteoblastic cells, at day 21.

Thus, the present results showed that the best osteoblastic behaviour was achieved with the cements filled with the silicate glass. These results might be related with the good cell response to the silicate containing materials, as silicon plays an important role in bone physiology, having a positive effect on osteoblasts [48]. The silicate glass has also a low dissolution rate, as shown by the WU, WL and SEM assays, which creates a stable surface for cell adhesion and proliferation. Comparatively, as referred above, the cements filled with borate glass presented a high dissolution rate, as evident by the WU and WL values, creating an unstable surface with very dynamic dissolution/deposition events that impairs cell growth. The presence of the polymer PHB or PHBV also affected significantly the cell response. In this way, the inclusion of the polymer PHBV greatly improved the osteoblastic cytocompatibility, both in the cements filled with the silicate or the borate glass, compared to the inclusion of the polymer PHB. In the cements filled with PHB and borate glass, in addition to their high degradation rate, the higher amount of residual monomer found in these cements, released to the culture medium due to the dissolution events, might also contribute to the poor biological performance, because of the known toxicity of the monomer [49]. However, *in vivo*, it is believed that the deleterious effect of ion and monomer release might be greatly attenuated, due to the continuous circulation of the body fluids and the resulting clearance from the regeneration area, improving the cell response.

CONCLUSION

The comparative study of PHB and PHBV-containing cements evidenced the high performance of PHBV filler regarding mechanical properties and biocompatibility. Considering the setting parameters and comparing with the non-filled PMMA matrix, the behaviour was quite similar for both cements, namely a lower peak temperature and a longer setting time, which might represent a extended time for bone cement preparation/application during surgical procedure.

The thermal analysis revealed a decrease in the melting temperature and in the degree of crystallization when PHB was added to the cements, probably due to a distortion of the lattice caused by the presence of MMA.

In term of mechanical properties, the incorporation of PHBV led to higher bending strength and similar elastic modulus, when compared with PHB filler reaching appropriate values to be used as bone cements. The most relevant values of bending strength were obtained for the lower biodegradable filler concentration (10 wt.%). The cements filled with PHBV also showed the highest weight loss at 21 days and water uptake at 60 days. The formation of a calcium phosphate layer was verified for all investigated cements, demonstrating that they are potentially bioactive.

Finally, the results demonstrated that PHBV-containing cements promoted a more developed and well-organized osteoblastic cell layer and achieved higher cell proliferation and ALP activity, indicating that the inclusion of this polymer resulted in a better biological response compared to that observed on the cements containing PHB.

Acknowledgements

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CHAPTER 6

CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

CONCLUSIONS

Acrylic bone cement has emerged as one of the most used synthetic biomaterials in contemporary orthopaedics being used in the treatment of bone defects and fixation of implants.

Although universally used for many years acrylic bone cements are beset with a number of drawbacks that limit their performance such as non-bone-bonding capability, relatively low mechanical strength, release of unreacted monomer and high curing temperatures. These problems can cause serious complications in vivo, such as necrosis of the surrounding tissues and even loosening of the implant.

This thesis was conceived to be a contribution for the development of an improved formulation of bone cement, aiming at solving some of the known drawbacks of the conventional bone cements, as aforementioned. Our approach to achieve such objectives consisted in developing a bioactive bone cement varying the composition of its solid phase, in which the PMMA was replaced by bioactive glasses.

The initial strategy was to produce composites filled with a silicate glass (CSi) and a borate glass (CB), by thermal route, and to compare their in vitro performance in acellular and cellular media. Both bioactive glasses, added to the formulations, allowed the formation of a calcium phosphate layer. This layer is a strong indication that, when in vivo the cement is able to bond directly to bone. The bioactive fixation would constitute the main mechanism of adhesion to the bone avoiding the appearance of the fibrous tissue layer and preventing the occurrence of micro movements. The cellular assessment resulted in a dissimilar behaviour of the composites, in which only CSi demonstrated an inductive effect on the proliferation of cells. The results showed that the high B and Mg ionic concentration in the cell culture medium inhibited cell proliferation on the CB.

Commercial bone cements are prepared in the operating room and the polymerization of MMA occurs at room temperature initiated by a redox system consisting of a peroxide (initiator) and tertiary aromatic amines (activator). So, most of the experimental part of the work is based on this method of polymerization using the chemical route and producing self cured cements. Practically all commercially available formulations of cements use N,N dimethyl-p-toluidine (DMT) as activator, which is known to be a potential carcinogen, a chromosome-damaging agent and an

inhibitor of protein synthesis. Within this context our choice was to introduce an activator of reduced toxicity (4,4-bis-dimethylamino benzydrol) in the cement preparation, in order to obtain cured materials with improved biocompatibility.

The effect of glass content (30, 40 and 50%) and composition of filler (silicate glass or borate glass) in self cured cements was assessed. The addition of bioactive glass filler exhibited significant improvements in the curing parameters and in the mechanical properties of the cements (bending and compressive). These properties are more dependent on the filler concentration than on the glass composition. The composition of the glasses was relevant for the osteoblastic cell response, since the incorporation of the silicate glass significantly improved osteoblastic cytocompatibility, whereas the presence of the borate glass resulted in a poor cell response. Nevertheless it was demonstrated that the surviving cells on the B-glass cement surface were in a more differentiated stage compared to those growing over the non-filled PMMA.

Loosening of the cemented prostheses results not only from the failure of the implant and/or the bone cement, but also from the inflammatory response of the bone tissue against bone cement ingredients. Thus, in a part of this work it was explored the possibility of incorporating ibuprofen (anti-inflammatory drug) into the cement, aiming to have a material that shows simultaneously controlled drug release and bioactive behaviour. Different amounts of ibuprofen (5, 10 and 20 wt.%) were loaded in the bioactive bone cement (cement filled with silicate glass, 50 wt%). The curing parameters of the bioactive bone cements were improved with the increasing amount of ibuprofen leading to a reduction of the peak temperature and an extension of the setting time. It was also investigated whether the developed cements had the suitable effect to potentiate the required therapeutic action in a real situation. The compositions IB5 showed insufficient daily dose of release for the treatment of inflammation. The IB10 and IB20 cements achieved the sufficient therapeutic effect for the treatment of inflammation for one week and two weeks, respectively.

Considering the bioactive bone cement, a significant part of the bioactive glass particles are covered with the polymeric PMMA matrix and the contact with the physiological solution is absent, i.e. only the particles at the cement surface are accessible, restricting the formation of calcium phosphates associated to mineralization. Definitely a much stronger interaction should be attained if the bone was induced to grow also inside the cement. This strategy can be achieved with the introduction of biodegradable polymers in the bioactive cement formulations, suggesting that bone could be stimulated to grow around, as well as into the cement inside the space left by the degraded material resulting in a stronger fixation of the prosthesis.

In the final study conducted in this work the possibility of incorporation of polyhydroxyalkanoates (PHB and PHBV) and a bioactive glass into the bone cement, combining a biodegradable and a bioactive filler was explored. The results evidenced the high performance of

PHBV polymer regarding mechanical properties and biocompatibility. Concerning PHB a reduction in its degree of crystallization was verified due to the presence of MMA, which can be responsible for the lower mechanical properties and impair cell response. The used polymers have different sorption abilities of the monomer because of differences in their particle size. Accordingly, PHBV-containing cements promoted a more developed and well-organized osteoblastic cell layer and achieved higher cell proliferation and ALP activity. The data herein presented demonstrates the potential and versatility of the proposed material for improving the cement performance.

SUGGESTIONS FOR FUTURE WORK

Despite the extensive *in vitro* characterization addressed in this thesis, a research work is never completed. Thus, it would be beneficial, in our opinion, to concentrate on the following issues in future research.

Several aspects of the fatigue behaviour of developed cement should be investigated, since the bone cement is also subjected to cyclic loading *in vivo* and its fatigue properties are of great importance to experimental testing and clinical performance.

Porosity is the key feature involved in mechanical failure and damage accumulation. Optimized mixing methods such as vacuum-mixing and centrifugation should be tested to evaluate its effect on the mechanical properties of bone cements.

The addition of radiopaque agent to the cement and the evaluation of its effect on the curing parameters, mechanical properties, *in vitro* degradation and biocompatibility of the materials should be an interesting focus of study as well.

The study of the cement as a drug delivery system should be extended to the incorporation of antibiotics like vancomycin and gentamicin, which can be used to prevent and treat periprosthetic joint infections.

Finally, in a further stage, *in vivo* experiments should be performed, which would confirm whether the cement has adequate properties for the intended applications.