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Developments in injectable multiphasic biomaterials. The performance of microporous biphasic calcium phosphate granules and hydrogels

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Abstract Calcium phosphate bioceramic granules associated with hydrosoluble polymers were developed as bone substitutes for various maxillofacial and orthopaedic applications. These injectable bone substitutes, support and regenerate bone tissue and resorb after implantation. The efficiency of these multiphasic materials is due to the osteogenic and osteoconductive properties of the microporous biphasic calcium phosphate. The associated hydrosoluble polymers are considered as carriers in order to achieve the rheological properties of injectable bone substitutes (IBS). In this study, we used 2 semi synthetic hydrosoluble polymers of polysaccharidic origin. The hydroxy propyl methyl cellulose (HPMC), with and without silane, was combined with microporous BCP granules. The presence of silane induced considerable gelation of the suspension. The 2 IBS used (without gelation, IBS1, with gelation, IBS2) were implanted in critical size femoral epiphysis defects in rabbits. No foreign body reactions were observed in either sample. However, because of the higher density from gelation, cell colonisation followed by bone tissue ingrowth was delayed over time with IBS2 compared to the IBS1 without gelation. The results showed resorption of the BCP granule and bone ingrowth at the expense of both IBS with different kinetics. This study demonstrates that the hydrogel cannot be considered merely as a carrier. The gelation process delayed cell and tissue colonisation by slow degradation of the HPMC Si, compared to the faster release of HPMC with IBS1, in turn inducing faster permeability and spaces for tissue ingrowth between the BCP granules.

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1 Introduction

There are numerous clinical indications in which materials are needed to restore and regenerate bone and the best material is autologous bone graft, but numerous limits have been described in the literature [1–3] The ideal synthetic biomaterial should be injectable and mouldable, should set in the defect, and should favour bone apposition and growth while being degraded by body fluids and by cells. Ultimately, the material should be replaced by mature bone tissue within a healing period of weeks.

Our approach has focused on multiphase biomaterials that associate bioceramics and hydrogel in order to replace and regenerate bone tissue in osseous or dental defects. This multidisciplinary research involves solid state

chemistry of calcium phosphate bioceramics, organic chemistry with synthetic polymers, and animal models for testing biocompatibility and bio-functionality.

Our approach has focused on multiphase biomaterials that associate bioceramics and hydrogels as a method for replacing and regenerating bone tissue in bone or dental defects. This multidisciplinary research involves the solid state chemistry of calcium phosphate bioceramics, organic chemistry with synthetic polymers, and animal models for testing biocompatibility and bio-functionality.

The concept of biphasic calcium phosphate (BCP) ceramics, mixing HA and β-TCP in various HA/β-TCP ratios, has been widely developed by Daculsi and Legeros since the 1990s [4, 5]. Bioceramics have open macropores in the 100–600 μm range and micropores in the 0.1–10 μm range in order to allow the penetration of body fluids, cells, tissues and vascularization. The BCP concept [6] is based on an optimum balance between the more stable phase (HA) and the more soluble phase (β-TCP). BCP bioceramics are soluble and gradually dissolve in vivo, seeding new bone formation as it releases calcium and phosphate ions into the biological medium. The formation of the dynamic interface between bioactive ceramics and host bone is believed to result from a sequence of events involving interaction with cells and the formation of carbonate hydroxyapatite CHA (similar to bone mineral) by dissolution/precipitation processes. Bioceramics support bone formation while they partially dissolve and degrade in the body.

The development of minimally invasive surgery (MIS) requires the development of injectable bone substitutes. The BCP granule concept has been applied in the development of these new generations of injectable, mouldable bone substitutes [7]. BCP granules are combined with various polymers—natural (e.g. fibrin sealant), or synthetic (e.g. hydrosoluble polymer)—for the development of either injectable bone substitutes (IBS) [8], or calcium phosphate cements that improve macroporosity and provide greater osteoconduction [9]. Hydrosoluble polymers, such as the HPMC used in this study, are considered to be merely carriers for BCP granules. The CaP particles are suspended in physiological fluids containing hydrophilic polymers [10]. The viscous solution promotes the injectability of the BCP granules, without phase segregation. However, this suspension remains viscous. To maintain the suspension in an unclosed cavity and prevent it from being washed out by biological fluid, gelation is an advantage. For this development, HPMC with silane was proposed [11–13]. Gelation, however, can modify both the diffusion of biological fluids and cell colonisation. The purpose of our experimental study was to determine the influence of two suspensions on cell invasion and bone ingrowth in critical size bony defect in rabbits. The two suspensions were IBS1, HPMC with granules but no gelling properties, and IBS2, HPMC Si with granules and gelling by cross-linking caused by silane grafting.

2 Materials and methods

2.1 Raw materials

- BCP granules (Biomatlante SAS, Vigneux de Bretagne, France) are a mixture of hydroxyapatite (60%) and β-TCP (40%) with 25% ± 8 micropores of less than 10 μm in diameter. The granule size was of 80–200 μm, with a mean size of 120 μm in diameter.
- HPMC (hydroxypropyl methyl cellulose) is a semi synthetic polymer derivative of polysaccharidic origin. The HPMC hydrogel was prepared in 3% isotonic solution.
- HPMC-Si was a silated HPMC obtained by grafting silane with HPMC [10, 11]. Briefly, the HPMC-Si hydrogel was produced by dissolution of HPMC in NaOH solution at a pH equal to 12.8 and 3-GPTMS (3-glycidoxypropyltrimethoxylane.) as silated agent. Extemporaneously, a buffer solution was mixed with the polymer solution to obtain a final pH ranging from 7.4 to 8.

2.2 Suspensions

IBS1 (MBCP Gel®, Biomatlante SAS) was prepared with 40% in weight of BCP granules (mean 120 μm) mixed with HPMC hydrogel (3% solution). The suspension was contained in a syringe, and steam sterilisation was performed. IBS1 is a ready to use, non hardening, injectable biomaterial. IBS1 has rheological properties capable of ensuring that the mineral phase bonds in situ and that it has high permeability and suitable rheological properties for an 18-gauge needle. MBCP gel® has the CE label and FDA approval, and was used as a reference for the bioceramics suspension in comparison with the IBS2 used in this study.

IBS2 is a self-hardening composite associating BCP granules (the same as in IBS1) with a silated hydrogel, HPMC-Si. The hardening of the hydrogel is the result of a cross-linking reaction between the silane groups bound to the cellulosic polymer. The silated hydrogel/calcium phosphate composite induces self-reticulation obtained with the pH change as a catalyst without exothermal effect [14, 15]. Prior to cross-linking, the composite is an injectable viscous liquid, that hardens in the bone defect, forming a gel loaded with BCP ceramic particles. In this study, no purification using dialysis processes was performed to reduce free silane [16]. The IBS2 was prepared mixing 4.1 g of BCP granules in 4 g of HPMC-Si at 2.5%

in a NaOH 0.2 M solution. The mixture was steam sterilised. Before filling the defect extemporaneously, the composite was mixed with 1 ml buffer at pH 1.4 to obtain a pH for the composite of around 7.8. The mixture was mixed under sterile conditions for 1 min, and the bone defect was filled using a syringe.

3 Animal experiments

Animal handling and surgical procedures were conducted according to European Community guidelines for the care and use of laboratory animals (DE 86/609/CEE) and approved by the ethics committee of the Veterinary School.

Eighteen female adult New Zealand White rabbits (Charles River, Saint Aubin les Elboeuf, France) were used. Three groups of six rabbits were formed (IBS1, IBS2, HPMC Si alone). Under general anesthesia performed by intramuscular injections of xylazine (5 mg/kg) and ketamine (35 mg/kg), bilateral femoral implantations were performed in aseptic conditions. A cylindrical bone defect (6 mm in diameter and 10 mm deep) was created at the distal femoral end and rinsed with physiological saline solution. The defects were filled with one of the composites and carefully compacted to prevent the formation of dead spaces.

Intramuscular paravertebral implantations were performed with the 3 implants. The implantation sites were labelled using a surgical suture clip. Under general anaesthesia, the animals were sacrificed 6 and 12 weeks after implantation by means of an intracardiac overdose of a barbiturate (Dolethal®, Vetoquinol, France).

4 Analysis

The implants were harvested and fixed in neutral buffered formalin solution for 48H, for histomorphometrical analysis of the trabecular bone. After fixation, the samples were embedded in polymethylmethacrylate (PMMA) resin. Each block was cut in half with a circular diamond saw (saw microtome 1600, Leica, Germany). One part was processed for histology while the other block was used for histomorphometrical measurements. Sections measuring 100 μm were made and observed with a polarized light microscope. Thin, 7 μm sections were then prepared with a hard tissue microtome (Reichert-Jung, Supercut 2050, Germany) and stained using Movat's pentachrome.

SEM observations were made using secondary and back-scattered electrons (BSE) at 15 kV (Leo 1450VP, Zeiss, Germany). A quantitative evaluation of the mineralized bone, MBCP granules and cellular areas was performed using an image analysis system (Leica Quantimeter). All data were expressed as averages and standard error. Differences were evaluated by analysis of variance (ANOVA) with Fisher's probability least significant difference (PLSD) post-hoc test. Differences were considered significant for $P < 0.05$.

5 Results

The density of the BCP granules in IBS 1 and IBS 2 before implantation was 49% ± 2.

After implantation for both materials, no clinical sign of rejection or major inflammation was observed, in either the bony sites or the muscular areas.

5.1 Muscle implantation site

The harvested HPMC Si alone implants were difficult to find in the muscle after 6 weeks, and very difficult or impossible to find at 12 weeks in spite of the presence of the suture clip.

For IBS 1 and IBS 2, no ectopic bone formation was observed, and there we have not observed mineralised matrix. Only cellular soft tissue occupied the spaces between the BCP granules, with numerous multinucleated resorbing cells (macrophages) at their surface; no fibrous encapsulation was observed in either sample.

For HPMC Si alone, monocytes, macrophages and multinucleated giant cells were observed surrounded by

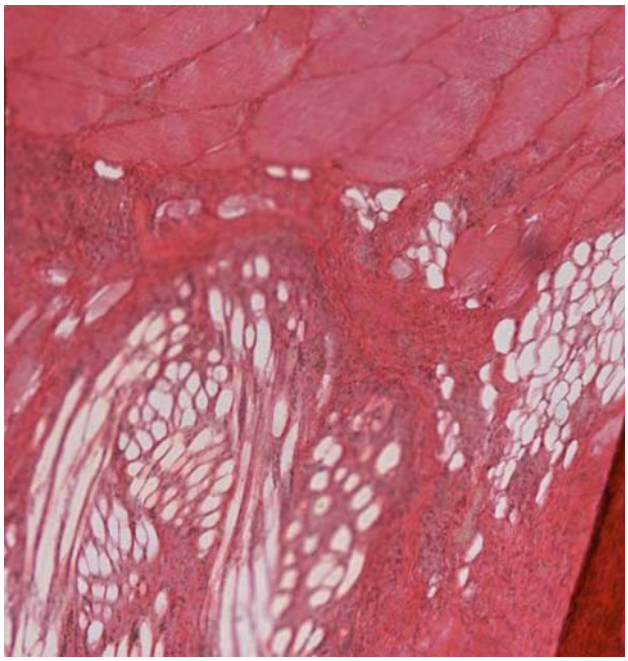

Fig. 1 HPMC Si alone implanted in lumbar muscle (M) after 6 weeks (arrow) surrounded by numerous macrophage and giant cells. Light microscopy, Movat's staining

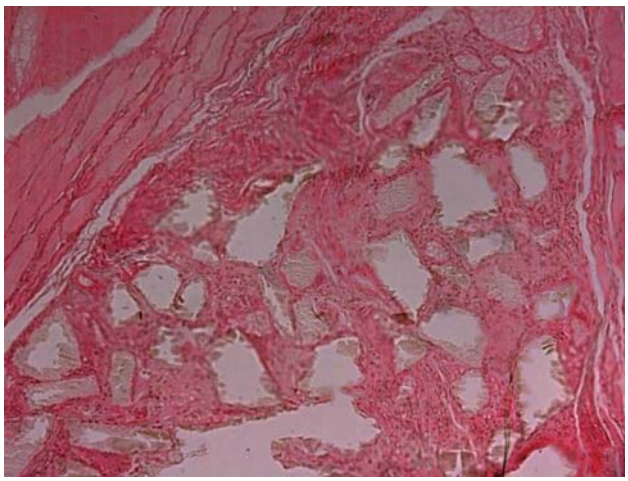

Fig. 2 IBS1 implanted in lumbar muscle (M) after 12 weeks. BCP particles (arrow) surrounded by numerous macrophage and giant cells. Light microscopy, Movat's staining

fibrillar tissue (Fig. 1), and after 12 weeks there were no significant traces of the implant remaining, the polymer had been totally resorbed, and no signs of any residual foreign body could be observed. For IBS 2, we observed the same macrophage content and multinucleated giant cells (Fig. 2). At 12 weeks, greater resorption of the granules was maintained, and contrary to IBS1, the IBS2 showed a dense fibrillar network all around the implant site.

5.2 Bony implantation site

At 6 weeks, IBS1 showed complete colonisation by living tissue, and monocytes and macrophages were observed contributing to considerable resorption of the granules (56%). Bone ingrowth appeared from the host bone to the core of the

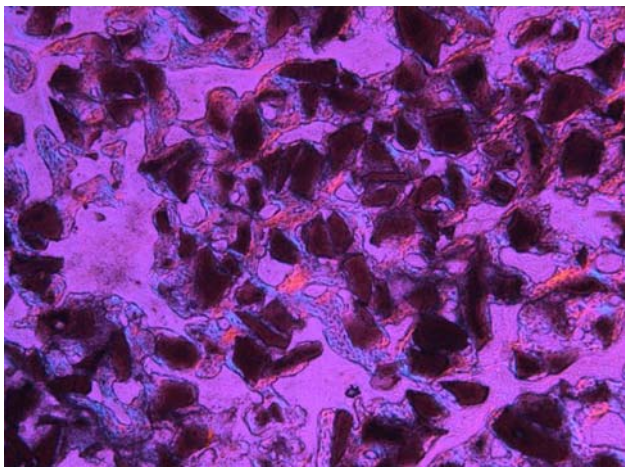

Fig. 3 IBS1 implanted in femoral epiphysis after 6 weeks. BCP particles (black) surrounded by interconnected bone trabeculae. Polarised light microscopy

Table 1 Image analysis of the residual BCP granules and newly formed bone after implantation in rabbits

	Time implantation	% Granules	% Newly formed bone	% Soft tissue
IBS1	6 weeks	21.4 ± 1.7	28.6 ± 8.8	50.0 ± 12.6
	12 weeks	15.3 ± 1.6	39.1 ± 5.2	45.6 ± 4.7
IBS2	6 weeks	30.1 ± 1.3	9.1 ± 9.4	40.8 ± 2.3
	12 weeks	21.5 ± 1.5	8.6 ± 4.1	69.8 ± 2.7
HPMC	6 weeks	–	1 ± 1	99 ± 1
	12 weeks	–	2 ± 1.5	98 ± 2

Density of trabecular bone epiphysis: 29% and soft tissue 71%

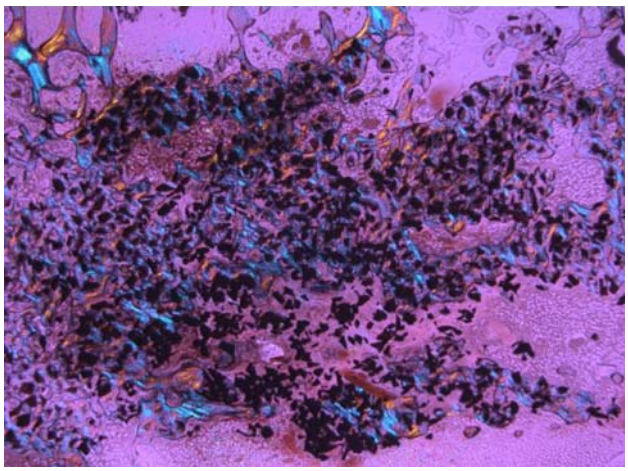

Fig. 4 IBS1 implanted in femoral epiphysis after 12 weeks. BCP particles (black) surrounded by interconnected bone trabeculae. Polarised light microscopy

implant (Fig. 3). Histomorphometry data for the residual BCP granules and bone ingrowth are reported in Table 1. At 12 weeks, greater resorption and bone ingrowth was observed and measured (69%). The scaffold effect of the residual BCP granules was highlighted, and numerous residual granules were integrated into the bone trabeculae (Fig. 4).

For IBS2, at 6 weeks cell colonisation was limited to the interface with the host bone and was not in the core of the implantation site (Fig. 5). In spite of the large number of macrophages and multinucleated giant cells, the resorption of the particles remained limited to 39%. Few bone trabeculae were observed and were limited to the external surface of the implantation site. At 12 weeks, more cells and soft tissue were observed between the intergranular spaces of the implantation site (Fig. 6). Bone trabeculae had invaded some spaces, but no regular distribution through the implant site as a whole was observed, and the BCP granules had only a limited scaffold effect (direct bone contact with granule surfaces). The percentage of granule resorption was 56%. The surfaces of the granules were covered mainly with resorbing cells.

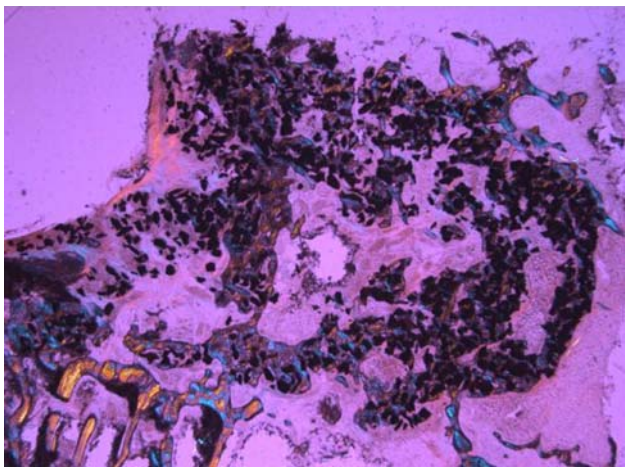

Fig. 5 IBS2 implanted in femoral epiphysis after 6 weeks. BCP particles (black). Bone trabeculae limited to the periphery of the implant. Polarised light microscopy

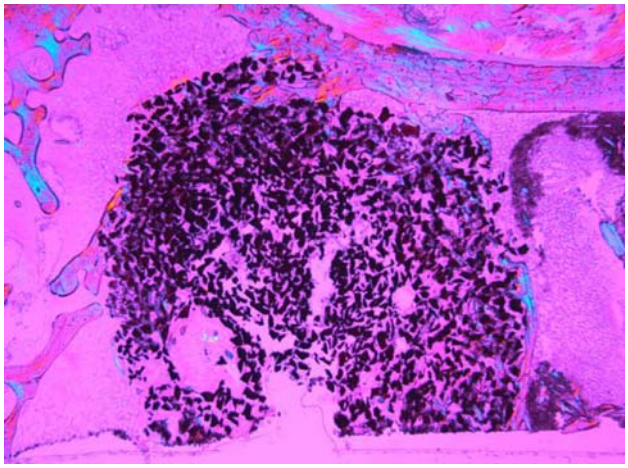

Fig. 6 IBS2 implanted in femoral epiphysis after 12 weeks. BCP particles (black). Polarised light microscopy

For the defect filled with HPMC Si alone, no bone formation or non mineralised soft tissue could be observed at either 6 or 12 weeks. The defect was filled only with soft tissue (Fig. 7).

6 Discussion

To date, several injectable biomaterials have been developed. These injectable bone substitutes are made generally of CaP hydraulic cement that hardens in the bone defect [17, 18]. Others are composed of CaP granules suspended in hydrogel.

The biocompatibility and no foreign body reaction are recognized properties of hydrogels based on HPMC [19, 20]. The use of silane for cross-linking improves the

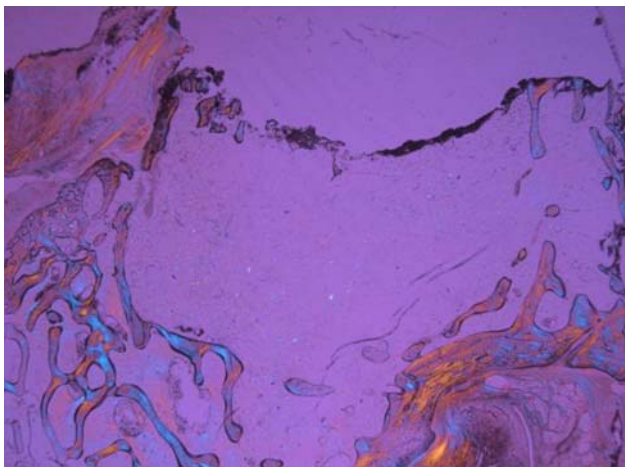

Fig. 7 HPMC Si alone implanted in femoral epiphysis after 12 weeks showing no organised connective or bone tissue growing in the site of implantation. Polarised light microscopy

rheological and mechanical stability of the injectable bone substitute [21], preventing washout in biological fluid. In under particular inhalation conditions it has been shown that free silanes can have cytotoxic effects [22]. However in orthopedic cement containing silane, after in vivo implantations, no cytotoxicity has been reported [23]. It is nevertheless recommended that dialysis high purification methods must be used. In spite of the fact that there was no dialysis in this study, we did not have any reported allergic, high inflammation reactions in either the muscular area or the bony site of our IBS2 preparation. A previous study carried out by our group did use dialysis purification of the HPMC-Si [24], and we reported similar results in terms of no clinical signs of an allergic effect. However, the bone ingrowth measurements made at 8 weeks were different and higher than those in the results from this study. The difference will doubtless be found in the composite's manufacturing process, and the IBS2 in the present experiment was ready to use, with the BCP granules and HPMC-Si previously mixed with an industrial mixer and sterilised before storage and use. In the previous study [24], the BCP granules and HPMC Si were sterilised independently and mixed manually extemporaneously. The other changes between the two experiments were the buffer composition and consequently the final pH, the size of the BCP granules (40–80 μm and 80–200 μm) and finally the presence of free silane in the present study (dialysis) which can change the bioactivity. The last possibility for explaining the difference in biological colonisation may be, in these two experiments, the interaction and steam effect of the mixture with the medical devices used in the industrial process and the extemporaneous preparation. A strong interaction has been reported between the HPMC and BCP surface [25] at the unit cell level with HPO_4

integration into the lattice. These interactions at the nanoscale surface may have an influence on osteoconduction and osteogenic properties. However in this study we have not explored if the wetting of the particles is the same with and without silane, or if discontinuities or flaws at the polymer ceramic interface were equivalent between the 2 kinds of hydrogels.

The first generation of injectable bone substitute IBS1 (MBCP Gel®, Biomatlante SA) is a non hardening injectable biomaterial. It consists of BCP granules in suspension associated with a hydrosoluble polymer. IBS1 requires rheological properties capable of ensuring bonding of the mineral phase in situ with good cell permeability. This original biomaterial is produced in a sterile ready-to-use cartridge form. Its composition and properties are suitable for a reproducible biological response. Since the product is not prepared during surgery, the risk of infection is minimal. IBS1 is biocompatible and potentially resorbable. Its initial plasticity makes it possible to fill complex-shaped bone defects very easily. IBS1 does not have as much mechanical strength as hydraulic calcium phosphate cements, but it leads to rapid and abundant bone growth thanks to its intrinsic porosity. Bone cells are able to invade the spaces created by the disappearance of the polymer carrier. Bone ingrowth takes place all around the granules at the expense of the resorption of the BCP granules.

The second type of injectable bone substitute (IBS2) is a self-hardening composite. The BCP granules are associated with the silanised hydrogel, HPMC-Si. The hardening of the hydrogel is the result of a cross-linking reaction between silane groups bound to the cellulosic polymer. The silated hydrogel/calcium phosphate composite involved self-reticulation obtained with the pH change as a catalyst and with no exothermal effect [13–15]. Prior to cross-linking, the composite is an injectable viscous liquid that hardens in the bone defect, forming a gel loaded with BCP ceramic particles. IBS2 can entirely fill and remain in bone defects. The BCP particles provide bioactivity supporting the bone healing process by means of osteoconduction. The cross-linked HPMC-Si hydrogel creates inter granular spaces for bone ingrowth. In the previous study [24], after 8 weeks, bone had grown centripetally and progressed towards the centre of the defects. Bone, ceramic and non-mineralised tissue were 24.6 ± 5.6, 21.6 ± 5.8 and $53.7 \pm 0.1\%$, respectively. Mineralised and mature bone could be observed between and in contact with the BCP particles. In the present study, after 12 weeks we had less bone ingrowth. The differences between the 2 studies could be the interaction between the HPMC Si and the calcium phosphate over time during storage and sterilisation of the IBS2, compared to extemporaneous preparation. The surface of the calcium phosphate and gelation before blood diffusion delay osteoconduction and cell and tissue colonisation over time at the expense of the composite. Further experiments are required to determine the BCP surface interactions with HPMC Si at the unit cell crystal level.

Gelation is an important property for preventing the injectable bone substitute from being washed out into the bleeding site after implantation, and for maintaining the bioceramic granules on site in unclosed cavities. However, the cross-linking increases the density of the material, reduces the dissolution or degradation of the polymer, and delays the diffusion of biological fluid and cell colonisation. Similar results have been described with fibrin glue combined with BCP granules [26], hydrogel cross-linked by irradiation [27], or in calcium phosphate cement with fast or long (over 45 min) setting times on cells and tissue ingrowth at the expense of the implants [28].

7 Conclusion

For clinical applications of injectable or mouldable bone substitutes such as composite hydrogel-BCP, it is necessary to be able to control the setting or cross-linking time after implantation with regard to the initial properties required:

For the initial mechanical stability, prevention of washing out, and initial mechanical properties, we needed setting/cross-linking, but as a result we had delayed resorption and bone ingrowth.

For closed defect cavities, implant resorption and fast bone ingrowth with secondary mechanical properties, we need a hydrosoluble polymer, with no cross-linking, or at least with fast degradation, such as polaxamer [29].

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