Acta Biomaterialia 9 (2013) 5421–5430

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Review

Hydrogels in calcium phosphate moldable and injectable bone substitutes: Sticky excipients or advanced 3-D carriers?

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ARTICLE INFO

Article history:
Received 12 September 2012
Received in revised form 16 November 2012
Accepted 19 November 2012
Available online 28 November 2012

Keywords:
Hydrogel
Calcium phosphates
Composite
Bone

ABSTRACT

The combination of hydrogels and calcium phosphate particles is emerging as a well-established trend for bone substitutes. Besides acting as binders for the inorganic phase, hydrogels within these hybrid materials can modulate cell colonization physically and biologically. The influence of hydrogels on the healing process can also be exploited through their capability to deliver drugs and cells for tissue engineering approaches. The aim of this review is to collect some recent progress in this field, with an emphasis on design aspects and possible future directions.

1. Introduction

As a consequence of trauma, disease and degeneration, the need for bone grafts is constantly growing. In 2010, the sales in bone graft substitutes were valued at $1.3 billion in the USA, with a forecast of $2.3 billion in 2017 [1]. Currently, the best results in terms of integration and new bone formation are obtained using autografts, featuring osteoinductive and osteogenic capabilities. The limitations of autografts consist mainly in explant site pain and morbidity, and limited availability [2]. Allo- and xenografts can overcome these limitations, but the risk of immune reactions and, in some countries, limited availability of tissue banks, patient compliance and regulatory restrictions are major hurdles at present [3]. In this respect, synthetic bone substitutes (SBSs) based on calcium phosphate (CaP) materials are valid alternatives to tissue transplants [4], and their clinical use dates back more than a century [5]. In the context of this paper, besides compounds synthesized via solution chemistry, thermal synthesis and thermal decomposition, CaPs also include semi-synthetic minerals such as deproteinized bovine bone.

In the 1980s and 1990s, only CaP blocks and granules were commercially available, while today all major suppliers include pastes and putties in their product portfolio [6,7]. Searching to assess the activities and interests in this area through the gathering of citations using specific keywords, a monotonous increase in publications on bone grafts since the 1990s was found, as well as a large number of studies on hydrogels (HGs) in the same period (Fig. 1).

Under the pressure generated by the established clinical need for improved bone grafts, the creation of optimal ceramic bone substitutes has been pursued, many design aspects being investigated. Literature on this subject is extensive, and has already been summarized in some remarkable reviews [4,8,9]. Besides focusing on refining inorganic phases, their combination with HG carriers is a viable method of improving CaP SBSs.

In the scientific literature, the lemma HG indicates both soft-jelly and hard though hydrated materials. Within the present paper, the term HG indicates soft gelatinous matrices whose rheological behavior features storage modulus prevalence over the viscous modulus ($\tan\delta \geqslant 1$, where δ is the phase shift angle between stress and strain).

The first elementary effect of the combination between HGs and granulates is the improved cohesion. The combination turns the brittle ceramic granulates into formulations that offer easier handling and moldability, making the products more appealing from the commercial point of view [7]. This is one of the reasons for the increasing popularity of the above-mentioned moldable pastes or putties. HGs are also used as carriers for the fabrication of injectable composites suitable for minimally invasive procedures.

Composites including bone substitutes and HGs are not a recent discovery. Fibrin sealants are significant examples of hydrated polymeric matrices used as carriers for bioceramics [10]. In spite of contrasting efficacy, the easy handling, good tolerability and surgeons' familiarity with these biomaterials have made them quite popular in bone reconstruction. Since their use has already been

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http://dx.doi.org/10.1016/j.actbio.2012.11.022

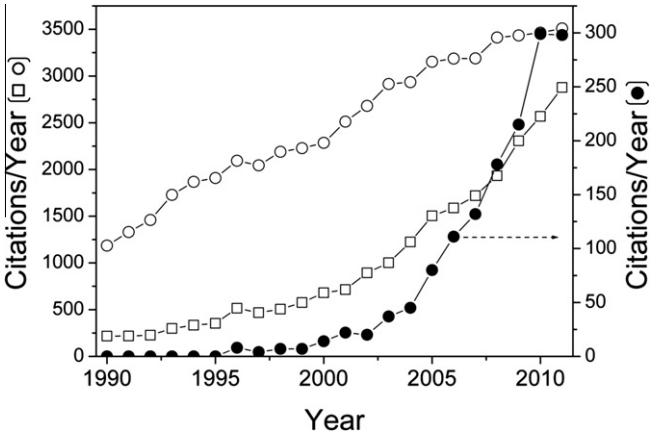

Fig. 1. Number of citations per year from 1990 to 2011 on bone graft (□) and hydrogel (○) and their combination (●) as keywords (literature search engine: www.scopus.com).

reviewed [11], fibrin sealant composites are excluded from this paper. In 1991, Ito [12] reported the "In vitro properties of a chitosan-bonded hydroxyapatite bone-filling paste". This early work was a formulation optimization aimed at obtaining an in situ setting paste with suitable mechanical properties and setting time, for the treatment of periodontal defects. In the mid-1990s, HG-based bone substitutes were already used in humans. Pompili et al. [13] reported several clinical cases of cranioplasty using a biomaterial consisting mainly of hydroxyapatite (HA) and gelatin (Osprogel, Tech-Medical S.R.L., Italy). According to the original article, based on a cohort of 11 cases analyzed, results were excellent in seven patients, good in three, and fair in one. At the same time, significant contributions on the association between bioactive CaP and hydrophilic cellulose derivatives were presented [14–16]. Noteworthy, despite the rapid expansion of tissue engineering in the early 1990s, the first significant reports on non-setting composites including cells were published only a decade later [17,18].

The improved cohesion provided by the HGs is certainly beneficial. However, HGs do not act as simple thickening excipients or carriers of osteoconductive ceramics. They provide a physical support, a degradable barrier and a hydrated three-dimensional (3-D) environment, which modifies the architecture of the cell–scaffold interaction, physically and biologically modulating the cell invasion into the construct, guiding the bone tissue regeneration accordingly.

Compared with autografts, which are at present regarded as the clinical gold standard, synthetic substitutes display lower osteoinductivity and osteogenicity. Next-generation SBSs are required to achieve these biological effects. As viable drug delivery systems for small effectors and biological drugs, HGs have the potential to cover this gap of bioactivity. Moreover, they provide hydrated architecture, and at times basic molecular building blocks for potent tissue engineering solutions.

The aim of this review is to collect some important contributions on combinations between fully or semi-synthetic ceramics and HGs. As the focus is on bone, other musculoskeletal tissues such as tendons, muscles and cartilage are not discussed. Cements are also excluded.

This paper is organized as follows. Section 2 focuses on design aspects, reporting how parameters such as particle size and gel rigidity influence phase stability, cohesion, tissue reaction, vascularization and in vivo bioactivity. Literature focusing on HGs as drug delivery systems for bone repair is already abundant, but the combination with CaP involves interesting peculiarities, which are considered in Section 3. Contributions more specifically related to the use of HG/CaP composites as cell carriers are discussed in Section 4. Finally, Section 5 summarizes some potential directions for future research in this field.

2. Composites based on HG and CaP materials: design aspects

The optimization of CaP porous bone substitutes has been the subject of countless studies over many years. In spite of these efforts, the design requirements for the optimal scaffold or synthetic bone graft are still unclear [4,8]. When porous materials are combined with HG matrices, an additional degree of complexity has to be included in the whole picture, and paradigms for optimal structure–property design of composite scaffolds are even more remote.

Bone tissue itself is a brilliant example of composite material consisting of nanocrystals of carbonated apatite spatially arranged within a hydrated collagen matrix, with considerable mechanical properties and intrinsic remodeling capability [19]. Therefore, the use of composites including CaP granulates embedded in an organic matrix is a mere imitation of the target tissue organization. Nonetheless, bone substitutes are not intended to reproduce the bone structure. They should promote the healing process, being finally replaced by functional tissue through remodeling.

2.1. Effect of granule particle size and composition

In HG/CaP composites, the HG part is often employed solely as a cohesive agent for the granules. Chemical composition and morphology of the CaP particles influence, among other things, the biocompatibility, mechanical properties, host tissue reaction and, finally, osteoinductive outcome. Certainly CaP and HG interact within the composite. The influence of (bio)polymeric matrices on the nucleation and mineralization of HA has been the subject of many investigations over the last two decades. It is well established that negatively charged groups such as the carboxy group attract Ca^{2+} ions, directing the HA crystals nucleation and growth [20–22]. HGs bearing positive charges display similar effect, attracting anions such as carbonate and phosphate [23]. From the solution chemistry perspective, CaPs are basically insoluble in water and chemically inert over a wide range of conditions. These features permit their inclusion within common HG matrices, avoiding the disruption of the polymer network. The chemical interaction of different biologically relevant CaPs (dibasic calcium phosphate dihydrate, calcium deficient apatite and biphasic calcium phosphate (BCP)) with a non-crosslinked hydroxypropylmethylcellulose (HPMC) polymer solution has been studied in stressing conditions (121 °C, 24 h). Within the sensitivity of the characterization techniques used (IR spectroscopy, X-ray diffraction and scanning electron microscopy), no sign of chemical interaction was detectable [24]. A composite of the same HPMC polymer with a BCP mineral phase was analyzed using high-resolution transmission electron microscopy [25]. In this case, evidence of hydrolysis via dissolution/precipitation was shown, but only from the surface to ~13 nm into the crystals.

Recently, Fedorovich et al. [26] reported ectopic bone formation for micro-sized BCP within Matrigel™ carriers, while unsintered apatitic nanoparticles embedded in the same HG only induced osteoclastic tissue response without bone formation. The results were attributed to the different particle size, crystallinity and degradability. The BCP used in the study was sintered at 1150 °C, while apatitic nanoparticles were produced in solution without any thermal treatment. The authors speculated that the lower crystallinity and higher surface area and therefore higher degradability of the nanoparticles were the reasons for the enhanced osteoclast activation observed.

Interestingly, using a similar animal model, Hulsart-Billström et al. [27] reported contrasting conclusions, with the density of the new bone higher for nanosized HA compared with micro-sized. In this case, the authors hypothesized that the higher bone density induced by nano HA might be due to the presence of nanocrystals of CaP acting as direct building blocks for biomineralization. However, in the latter paper, the HG carrier used was a bi-component in situ curing system supplemented with bone morphogenetic protein (BMP). The divergence of the conclusions is a reminder of how the outcomes of single studies depend on the specific experimental design and conditions, and most of the time are not sufficient to draw more general conclusions.

2.2. Rheological properties

HGs are macromolecular networks featuring high hydrophilicity and interconnections between the polymeric chains. This structure lets them swell without dissolving. Reticulations can originate from covalent bonds or weaker interactions, such as ionic, hydrophobic or hydrogen-bond forces, physical entanglement or microcrystallite formation [28,29]. They can present themselves already in the gel state or display gelation via crosslinking in situ (see Section 2.3).

The combination of CaP ceramic particles with polymeric carriers improves their handling within the surgical theater, and allows the formulation of injectable products for minimally invasive surgery.

The scarce mechanical properties and the non-applicability in load-bearing situations are often cited as limitations of HGs in bone tissue engineering. This is mostly a misconception. To date, none of the bone substitutes proposed in every possible form (including solid, elastomeric, putty and cement) are load bearing [8]. Therefore, when required, the mechanical support is provided by fixation devices [30]. Still, local mechanical changes in a bone defect due to the introduction of a bone graft can influence the bone healing response [31].

Oliveira et al. [32] characterized solutions of sodium carboxymethylcellulose, HPMC and sodium alginate as vehicles of HA microspheres. Rheological profiles of HA suspensions at 20% and 40% w/w and different polymer concentrations were studied both before and after heat sterilization, and the results were correlated with the observed injectability. Sodium alginate of molecular weight 85 kDa at 7.25% w:v concentration was the best HG candidate carrier in terms of composite injectability. Fatimi et al. [33] studied how the rheology of an HPMC carrier influences the rate of sedimentation in CaP suspensions. Different particle size, polymer concentration and effect of the sterilization have been analyzed. Sedimentation was found to follow Stokes' law qualitatively, with slower sedimentation promoted by smaller particle size and higher viscosity of the carrier. Interestingly, the spacer property of the hydrophilic carrier was speculated to have a positive effect on osteoconduction and bioactivity [33]. The rate of sedimentation is fundamental in terms of shelf stability of the devices and to avoid random migration of the particles after implantation, and unpredictable osteoconduction as a consequence [34]. In other applications, such as the reconstruction of bone structures such as facial bone, in order to keep the material in situ, an even higher stiffness is required. For this specific application, scaffolds with fixed shape rather than soft HG formulations may be preferred [35]. Phase stability is even more critical for injectable formulations, where the pressure gradient may induce separation between carrier and insoluble granulates. The parameters influencing injectability and filter pressing upon injection of CaP hydraulic pastes have been thoroughly analyzed [36–40]. Injectability is promoted by a high liquid to powder ratio, quicker extrusion, small syringe size and short cannula [36]. Recently, a model for the prediction of the extrusion force of CaP pastes was compared with experimental results from CaP suspensions in non-Newtonian HPMC solutions. While there was good agreement for the polymer solution, the model overestimated the force for the non-Newtonian suspension [41].

Besides phase separation, humid formulations have more issues of chemical and microbiological stability compared with dry ones. The sterilization method is therefore very important. All sterilization methods degrade the polymeric matrix to some extent; therefore rheological features have to be designed accordingly. Stability towards sterilization is even more critical when reactive chemical functionalities are included, as in the case of in situ curing systems. Moreover, while HGs are typically sterilized with saturated steam, SBSs may be compromised in these conditions. If different sterilization methods are needed for HG and SBS, aseptic filling is necessary, with massive cost increase as a consequence.

2.3. Composites based on in situ forming HG and CaP particles

Improved patient compliance and faster recovery make endoscopic techniques highly desirable approaches to treating bone defects. In situ forming HGs are generally regarded as ideal matrices for minimally invasive surgery. However, in the specific case of HG/CaP, the low viscosity required for easier injectability has a negative effect on shelf stability towards settling and phase separation upon injection. Moreover, as mentioned, the mechanical properties of the construct after curing in vivo have to be carefully designed, depending on the specific application. Of course, HG/CaP composites with in situ gelation display similarities with CaP cements because both display hardening once injected. The difference is that the final state is a gel for in situ gel-forming systems, while cements give rise to a solid from a paste or a semi-solid.

HG/CaP composites with in situ gelation triggered by chemistry that have recently been investigated include as matrices silated HPMC [42–44] and aldehyde-modified hyaluronic acid in combination with hydrazide-modified polyvinyl alcohol [27]. Silated HPMC is a HG self-hardening in a few minutes with non-exothermic reticulation promoted by the physiological pH exposure after injection. Its gelation has been deeply characterized, comparing the flow curves with theoretical models for different concentration, pH and temperature [45]. The hyaluronan/polyvinyl alcohol HG is instead a bi-component system, where the two components are stored separated and mixed during the extrusion from a specifically designed double-barrel syringe; CaP is pre-mixed at the same concentration in both pre-gel solutions. Among SBS composites with in situ gelation triggered by external stimuli, only thermoresponsive systems have been investigated. Mylonas et al. [46] used Pluronic® F127, while Lippens et al. [47] converted the hydroxyl end-groups of the same polymer into a crosslinkable *N*-methacryloyl-depsipeptide unit with tunable reticulation degree and degradation rate determined by the depsipeptide unit. For both contributions, a high concentration of Pluronic® ranging from 15% to 30% w/v had to be used in order to exploit the thermosensitivity of the polymer. Recently, Lin et al. [48] designed composites consisting of HA and a poly(ethylene glycol) (PEG) copolymer, namely poly(lactic acid-*co*-glycolic acid)–g-PEG. Interestingly, this is the only article reporting the viscoelastic profile as function of temperature. Concentrations of 30% w/w HG plus different amounts of HA were analyzed. For all the formulations tested, the storage modulus displayed a maximum at ~24 °C, dropping to values of <20 Pa at 37 °C. Therefore further studies are needed to optimize the mechanical properties of these composites. Still, thermoresponsive matrices are attractive carriers for CaP.

A different example of HG/CaP combination is the use of the gels for guided bone regeneration. This technique uses barrier membranes to direct the formation of the new bone tissue, keeping

the bone graft in place and excluding fast-growing fibrous tissues from invasion of the defect site. In clinical practice, preformed polytetrafluoroethylene membranes are mostly used for this purpose [49]. Humber et al. [49] applied an in situ forming PEG as a biodegradable membrane for guided bone regeneration in critical-sized parietal defects in rabbits. The voids were filled with a variety of graft materials, either in the presence of the guide or not. Authors reported migration of the membrane to occupy part of the defect volume. The effect was attributed to the fluidity of the membrane during application, and to the stress generated by closing and suturing the surrounding tissues. In a different model of acute standardized mandibular defect in dogs, Thoma et al. [50] used thiol-acrylate multiarm PEG chemistry to keep a deproteinized bovine bone mineral in place. Improved bone graft volume maintenance and ridge contour were reported over controls.

2.4. Interplay among gel rigidity, tissue response and new bone formation

After implantation, the cohesion at the site of implantation is of primary importance in order to avoid washing-out by body fluids. In this respect, in situ gelation is beneficial. However, support and cohesiveness are not the only requirements. Rigidity of the matrix and features of the granulate particles modulate tissue reaction, angiogenesis and, as a consequence, new bone formation. Sohier et al. [44] cultured ex vivo bone tissue engineered constructs, including BCP dispersed in a chemically crosslinked polysaccharide matrix of silated HPMC. The system displayed good viability, and osteoblastic differentiation potential was preserved, but cells did not proliferate. Thus, potential ingrowth of tissue in the SBS was impeded. The same group compared the crosslinked and non-crosslinked form of the same HPMC carrier in critical size femoral epiphysis defects in rabbits [43]. BCP granules (40% w/w) were dispersed in both matrices and injected after sterilization. The crosslinked carrier delayed cell colonization and new bone formation. Even though the silated HPMC matrix degradation without release of cytotoxic products had been shown [51], the results of the critical size femoral epiphysis defects study [43] were attributed to the reticulation and the non-ability of the cells to degrade and penetrate the construct in the appropriate time scale, as shown in vitro [44]. Similar outcomes were recently reported in an ectopic bone formation model in sheep [52]. In fact, Barbieri et al. [52] analyzed five different HG carriers for BCP granules. Composites were implanted in dorsal muscular pockets in sheep for 12 weeks, and the heterotopic bone formation was compared with the dissolution profile in vitro. BCP granules did not degrade within that time scale, but the general trend observed was improved bone formation into the gaps between the granules for HGs dissolving faster. The non-compliance of alginate within this trend was attributed to specific chemical features of that polymer. Ghanaati et al. [53] studied specifically the tissue reaction after subcutaneous implantation of β-tricalcium phosphate (β-TCP) as granulate or embedded into an HG composed of methylcellulose and hyaluronic acid. The implantation bed of the granulates was invaded by peri-implant tissue between day 3 and day 10. Conversely, for the paste formulation the influx of cells was limited, with a clear boundary between implant and surrounding tissues at day 3. Moreover, at day 10, the composite substitute displayed granulation tissue formation with the presence of multinucleated giant cells at the implant–tissue interface. Degradation of the implant was observed centripetally over a 60-day period. Furthermore, the polymeric matrix allowed the implanted mass to be less prone to dislodgment. The authors concluded that the presence of the HG matrix limited the fast connective tissue ingrowth into the gaps between the granules, and resulted in an optimal level of inflammation and better vascularization as a consequence.

Indeed, the degradation of the HG matrix is a fundamental aspect, and the techniques for studying this process are limited. Interestingly, Laïb et al. proposed a new method for labeling polysaccharides with a ruthenium complex suitable for X-ray microfluorescence detection [54].

Composite stiffness and degradation profile influence not only vascularization, cell influx or tissue reaction. In a recent study aiming to understand the influence of degradable scaffold on bone repair, Patterson et al. [55] prepared an HG with tunable degradation from methacrylated derivatives of hyaluronic acid photochemically crosslinked. Interestingly, they reported that the rate of scaffold degradation can also control the morphology of the newly formed bone, specifically affecting the organization of the collagen matrix with enhanced orientation.

To summarize, carriers featuring excessive rigidity and lack of degradation inhibit cellular colonization, preventing vascularization and fast bone ingrowth. The balance between mechanical stability and cell influx needs to be optimized as a function of the specific application (Fig. 2).

3. Composites based on HG and CaP as drug delivery systems

CaP intrinsic bioactivity is limited compared with autografts, and is insufficient in some cases. SBSs eliciting an improved biological response are therefore desired, and a few drugs and growth factors (GFs) in combination with bone graft substitutes have been explored. CaP graft substitutes have been loaded with drugs to elicit a faster bone repair, a lower bone turnover or for preventing infection [56–58]. Absorption on the surface of the inorganic phase has been the most described method for loading drugs onto biomaterials. The number of loaded molecules and their controlled release are then limited by the interactions with the topochemistry and surface area of the materials. While this remains a relatively simple method also for large-scale productions, the intrinsic disadvantages lie in suboptimal doses of the drug and possible loss of activity of the proteins.

However, the highly hydrated network of HGs is the vehicle of choice for the controlled spatial and temporal delivery of such factors [59]. Proteins and hydrophilic molecules are protected from denaturation and degradation in the hydrophilic polymer network, and their loading and release can be modulated by mesh size, degradation and specific interactions with the polymer [60]. Moreover, combining CaP particles and HG delivery vehicles could potentiate the modulation and control the release kinetic of multiple drugs, which may be crucial when considering the mode of action of GFs in bone repair.

3.1. Composites based on HG and CaP for the Delivery of BMP-2

Cytokines such as BMP-2 and BMP-7 (or Osteogenic Protein-1) have been the most widely studied GFs for improving bone healing [61,62]. Takaoka et al. [63] in 1991 and Okubo et al. [64] in 2000 demonstrated that local and controlled delivery of BMPs permitted a decrease in protein dosage and a reduction in ectopic bone formation. The type of carrier used to deliver the BMP has also been shown to be important for the modulation of the protein release and efficacy [65].

The literature on the preparation of HG/CaP composites with GFs (mainly BMP-2) is limited and little is known about their bone forming ability in vivo (Table 1). Selected HGs (e.g., hyaluronan, chitosan or PEG) have been employed in combination with CaP particles of different compositions and sizes. The HGs' ability to be combined with protein without denaturing it and the familiarity of the experimenters with the materials seem to be the main reasons for their choice. HG degradation and ability to support cell

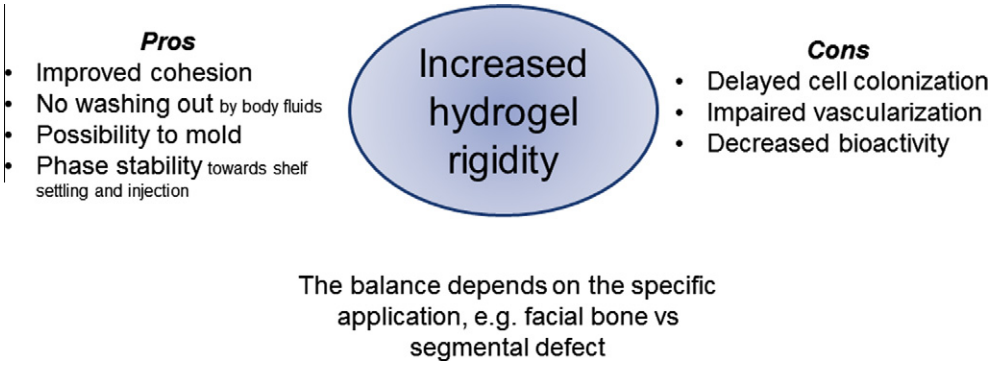

Fig. 2. Effects of increased matrix rigidity on HG/CaP composites.

Table 1
List of in vivo studies reporting efficacy of injectable or putty HG/CaP composites loaded BMP-2.

Author; year; reference	Inorganic phase	Polymer network	BMP2 ($\mu g\ g^{-1}$)	Type	In vivo model	Comments
Hulsart-Billström; 2011; [27]	β-TCP (45 μm) and HA ($0.02–10^2$ μm)	Hy–PVA	100–130	rh inductOS®	Rat; ectopic	Smaller HA particles was better
Docherty-Skogh; 2010; [67]	HA	Hy–PVA	250	rh inductOS®	Minipig; calvarial	Bone outgrowth
Luca; 2011; [72]	β-TCP 75–125 μm	Chitosan	750	rh inductOS®	Rat; ectopic; and rabbit; segmental defect	BMP2-TCP–chitosan was better in rat only
Jung; 2008; [66]	HA/TCP 0.5–1 mm	PEG–RGDS	10–30	rh	Rabbit; external calvarial	BMP2–HA/TCP–gel better than BMP2–gel
Tsuzuki; 2012; [70]	β-TCP 2 μm	Crosslinked gelatin	30	rh Peprotech Inc	Horse; metacarpal	Accelerate bone repair
Martínez-Sanz; 2012; [73]	HA 30 nm	Crosslinked hyaluronan	5–150	rh inductOS®	Rat; mandibular	Improved bone formation

β-TCP, β-tricalcium phosphate; HA, hydroxyapatite; Hy–PVA, hyaluronan–polyvinyl alcohol network; rh, recombinant human; PEG–RGDS, poly(ethylene glycol) functionalized with RGDS peptide 350 $\mu g\ g^{-1}$ polymer.

adhesion was also considered in some case [66]. The integration of GFs within CaP/HG composites is achieved via: (1) incorporation of drug into HG combined afterwards with a CaP phase [67]; (2) combination of the CaP with the drug, followed by incorporation into the HG [68,69]; or (3) addition of the drug into the premix composite [70]. To date, most in vivo studies (Table 1) reported BMP-2 loaded bone graft substitutes following the third methodology.

In vitro, the use of a PEG-based HG in combination with deproteinized bovine bone mineral resulted in a slower release and reduced activity of recombinant human BMP-2 (rh BMP-2) compared with the bone mineral carrier without HG [71]. The in situ HG chemical crosslinking mechanism (Michael addition) of the PEG was evoked as the reason for the deactivation of the loaded BMP-2, and the reduced efficacy in vitro. Thus, GF availability and activity are influenced by the design of the HG/CaP delivery system. In another study, Luca et al. [72] reported that an HG/CaP composite releasing BMP-2 induced more efficient bone formation compared with a HG releasing BMP-2. They hypothesized that the interaction between BMP-2 and β-TCP particles would slow its release from a chitosan HG, and a more sustained BMP-2 release would result in greater bone formation. The release profile of BMP-2 was not analyzed, but a higher bone mineral density (BMD) was reported in the BMP-2 loaded chitosan/β-TCP material compared with the HG control in an ectopic bone model. The difference in BMD obtained with the two materials was not significant when the initial β-TCP contribution was subtracted from the BMD value. Interestingly, the authors reported the presence of non-mineralized bone specifically within the BMP-2 loaded composite. Jung et al. [66] performed a similar experiment in the parietal and frontal rabbit bone cranium, using a modified PEG with HA/TCP granules loaded with two different doses of BMP-2. Reported quantitative histomorphometric results indicated that the presence of BMP-2 increased the fraction of newly formed bone compared with the empty defect and with the HG/CaP composite. The difference between the two doses of 10 and 30 $\mu g\ ml^{-1}$ was not statistically significant. Recently, Martínez-Sanz et al. [73] injected hyaluronan-based HGs containing nano-HA and different concentrations of BMP-2 into the subperiosteal space in rat mandibles. According to the procedure, the niche for the biomaterial was created by lifting the periosteum with the needle during the injection, without incisions and suturing. A dose-dependent increase in the mandibular bone volume was reported. Hulsart-Billström et al. [27] compared CaP materials of different compositions and particle size in BMP-2 loaded HG/CaP composites, with identical hyaluronan–polyvinyl alcohol HG as carrier. In an ectopic bone formation model in rats, nanoparticles of HA were found to induce higher bone density formation in comparison with larger HA and β-TCP. It was proposed that the inorganic phase and the BMP-2 loaded in the hyaluronan HG may have a synergistic effect on the amount of deposited new bone. The intrinsic bioactive properties of the specific particles, their ability to modulate BMP-2 availability and release, and their interactions with the HG matrix may be the cause of these findings. The dissimilarities in the amount of BMP-2 used and the in vivo models prevent the drawing of a general statement about the optimal design of composite drug delivery system. As recently demonstrated [74], the use of BMP-2 from different sources and different handling protocols are a major source of bias, too. In addition, when comparing healing of the same composite bone grafts loaded with BMP-2 in an ectopic and a segmental bone defect in vivo model, contrasting results were reported. While the ectopic model showed significant bone formation, the segmental defect did not heal completely [72]. In the

defect model, leakage of the material was observed, leading to uncontrolled ectopic bone formation. Thus, the most frequently used ectopic bone formation model does not seem to be adequate to probe and screen efficacy of the BMP-2 loaded composite bone graft substitutes in inducing healing in a difficult situation. This may be due to dosage issues, inter-species differences, compromised vascularization/tissue, and the mode of action of the BMP-2 in this case, which at high initial dose promote cell recruitment, while with sustained level of release encourages osteogenesis and angiogenesis.

The systematic comparison of the BMP-2 loaded composites, the CaP and HG materials loaded with an equivalent amount of BMP-2 (and unloaded materials) has not been published yet. A possible reason may be the difficulty in loading the BMP-2 at a similar concentration for all the materials and handling the materials in a comparable manner. Nonetheless, this would need to be performed in association with BMP-2 release experiments in order to clarify the role of each component and the interaction between components in these complex bone graft substitutes.

HG/CaP composites delivering BMP-2 have also been used in clinical trials for alveolar bone healing [75]. Seven patients with a mean age of 10 years suffering from unilateral alveolar cleft defect were randomized into three groups treated with autologous bone, composite with 50 $\mu g\,ml^{-1}$ of BMP-2 and composite with 250 $\mu g\,ml^{-1}$ of BMP-2. At the lowest dose, the implant was well tolerated, but new bone formation was almost absent after 6 months. At the highest concentration, the composite did induce significant bone formation, but the two patients in this group had significant gingival swelling during the first 2 weeks. The related co-morbidities brought the study to a premature conclusion. Interestingly, the authors used the identical compound (250 $\mu g\,ml^{-1}$) in a previous human study in neurosurgical adult patients without side effects (unpublished data). Pediatric patient might display enhanced sensitivity to the GF compared with adults. Still, the spatiotemporal control of the delivery provided by the matrix is essential and should be optimized, depending on the implantation site and avoiding side effects.

3.2. Delivery of other drugs from composites based on HG and CaP

Few other proteins and drugs relevant in bone repair have been loaded in HG/CaP composites: BMP-7 was delivered in vivo from a fibrin-loaded nanosized HA composite scaffold [76]; β-fibroblast growth factor was loaded in mineralized gelatin microspheres [77]; insulin-like growth factor 1 and TCP particles were dispersed in a gelling alginate matrix [69]; and enamel matrix derivate peptide was loaded in a composite matrix [78]. Parathyroid hormone employed for osteoporosis was also loaded in a PEG/BCP composite, but failed to demonstrate improvement in bone formation compared with the composite material alone in an in vivo model [79]. This again could reflect an inadequate release of the hormone and emphasize the need for a better understanding of the drug–HG–ceramic particle interactions and influence of preparation method in order to achieve a controlled delivery [80]. Another promising biological solution for bone tissue repair is the development of gene-activated matrices [81]. Indeed, a CaP nanoparticle carrier for DNA combined with an alginate HG allowed non-viral gene delivery in a controlled manner, avoiding the health risks associated with viral vectors [82]. The composite gene-activated matrices permitted the localized and efficient transfection of encapsulated cells with a BMP-2 plasmid in vitro.

Bone healing is a multistep process involving inflammation, angiogenesis, formation of callus and woven bone. Different GFs and cytokines are involved in each phase. Therefore, a potential improved scaffold for bone tissue regeneration could elicit different biological cues at different steps of the healing process [59,83,84].

Overall, a combination of CaP ceramic particles and HG as a carrier for GFs is a promising avenue for improving the efficacy of synthetic bone graft substitutes. Still, there is a need to improve understanding on the effect of HG/CaP-loaded drug or GF, whose value can only rely on better appreciation of the importance of the molecules' interactions with the composite and the need for relevant evaluation models.

4. Composites based on HG and CaP as cell carriers

The combination of ceramic particles, HG and cells for bone repair was studied as early as 2001 [17,18], but in these early reports the fibrin gel used as matrix was only considered as a binder for the inorganic phase and the cells. Since then, several combinations of HG and CaP particles have been reported as injectable cell carriers for the filling of bone defects. Different HGs have been used: gelatin, Matrigel™, PEG–fumarate, Pluronic®, alginate, chitosan, as well as CaP particles of several compositions (HA, β-TCP and BCP) and size (micron to nano) [46,85–89]. Mainly, mesenchymal stromal cells from mice, rat, rabbit, goat, dog and human, and an osteoblasts cell line (MC3T3-E1) have been encapsulated in composite bone grafts including HGs for their in vivo and in vitro assessment. Their phenotype, alkaline phosphatase production and mineralization, has been assessed mostly in osteogenic and growth mediums in vitro.

The fate of stem cells is directed by the matrix elasticity, with higher modulus fostering the commitment towards bone tissue [90,91]. In contrast, matrices with excessive rigidity display insufficient mass transport properties [92]. Therefore, the incorporation of CaP granules into HG matrices gives rise to a material combining a hard surface, to direct the bone tissue formation, and a water-rich matrix for transport properties, eventually supplemented with selected GFs.

Alginate HG, either unmodified or including collagen I, was reported to give scarce attachment and subsequent growth of human bone marrow stromal fibroblastic cells [93]. However, the simultaneous inclusion of β-TCP and type I collagen in the alginate HG enhanced adhesion and proliferation. However, the ability of the inorganic phase addition into the gel in inducing an improved biological response by surface interaction, Ca^{2+} dissolution or to act as a structural and mechanical element for the collagen and alginate was not discussed.

In a later report, a HPMC material and BCP particles encapsulating human bone mesenchymal stem cells maintained their viability and their osteogenic potential, as measured by alkaline phosphatase staining in a bioreactor in osteogenic medium [44]. However, inhibition of cell proliferation was observed, possibly due to the inability of the cells to first attach and then degrade the polymeric network, as already introduced. This evidence suggests that the HG matrix degradation and the ability of the cells to migrate within the matrix modulate the encapsulated cells' fate [94].

In most of the papers cited in this section, cells were added to a premix of the HG and the CaP particles. For example, bone marrow stromal cells were added directly in a premixed composite HG/CaP (silated HPMC and BCP of 60% HA and 40% TCP) within the gelling time [42,44]. In vitro, cell viability >95% was achieved in the HG containing up to 15 vol.% particles. However, addition of 30 vol.% particles led to a strong decrease in mesenchymal stem cells' viability, possibly produced by the grinding effect of the particle load in the gel and viscosity of the composition [44]. Few studies reported the initial seeding of the cells onto the inorganic particles before addition to the HG matrix [17,18]. Only one paper reports

a comparison between cells seeding on the particles vs. mixing within the composite, which resulted in different cell morphology and construct appearance at 5 weeks of culture time [44]. The seeding protocol has important consequences in defining the initial environment of the cells, either 3-D in the gel or 2-D on the microparticles, and determining their fate. Indeed, Mankani et al. [17] found an optimal BCP particle size range of 0.1–0.25 mm, leading to improved seeding efficiency and bone formation, while Trojani et al. [42] reported an optimal BCP particles size between 0.04 and 0.08 mm. The dissimilarity could be simply due to the cell encapsulation protocol or other multiple factors such as the cell type, the surface and shape of the particles or the type of HG matrix. Therefore, more comprehensive studies are required in order to fully exploit the potential of HG/CaP composites as cell carriers for bone healing therapies.

Of interest, a recent study reported the encapsulation of bovine chondrocytes into alginate–HA composites and the possibility of using this matrix to direct chondrocyte towards hypertrophy and mineralizing [89]. This could have an important impact in modulating and understanding bone healing mechanisms using mesenchymal stem cells encapsulated in a 3-D HG/CaP matrix.

In vivo, some studies reported better bone formation in the cell-seeded composite compared with the controls of composite and the gel without cells [18,42]. They both hypothesized the need for the resorbable organic phase and the cells to deliver the biological cues, the HG preventing the packing of particles, allowing vascularization and new bone tissue ingrowth. Although, HG aptitude to influence in vivo bone formation synergistically with CaP was reported recently [86]. Their combination with relevant cell sources such as mesenchymal stem cells has not yet been explored.

It could have been assumed that distinct applications such as cell-loaded HG/CaP, drug-loaded HG/CaP and standalone HG/CaP would require a specific range of HG/ceramic particles compositions. Compiling a series of 19 references with available HG/CaP preparation protocols, the results summarized in Fig. 3 were found. Polymer content values vary between 2% and 5% w/v for all the reported composites intended as cell, drug delivery and standalone, with the exception of a Pluronic® and a PEG HGs, 15% and 9% w/v, respectively. There is a very wide range of CaP content, with no specific trend between the groups. Finally, the CaP to polymer weight ratio ranges from 0.75 to 37.5, and the variance is higher for cell and drug delivery compared with the standalone composites. There is no general rationale underlying the compositions used in the different studies and applications compiled. In particular, the high amount of CaP used in some of the cell delivery systems seems counterintuitive, since shear and particle clogging during injection of a cell containing HG/CaP composites promotes cell death [44]. The lack of trends could be due to the dissimilar particle size, porosity and density of CaP used in the studies. Intrinsic viscosity and rheological behavior of different polymer matrices may have an effect, too. For example, in order to exploit the thermoresponsive properties of Pluronic®, the concentration used is about one order of magnitude higher (~15% w/v) compared with the other studies [47]. Therefore, development of new HG/CaP composites based on direct extrapolation from previous reports has to be avoided, as compositions are relatively material-specific rather than application-specific.

To summarize, cell therapy is a very promising tool for bone tissue regeneration. However, it should be remembered that its application in clinical practice is not straightforward. Cell therapy requires technically advanced facilities and has a huge inherent regulatory burden. If cells need to be isolated from the patient and expanded in vitro prior to delivery to the injured site, two surgical interventions are necessary. Cell therapies, like any other method in health care (e.g., pharmacological therapy or medical devices), need to be designed as simple, cost effective and profitable for the providers. This guiding principle should inspire the work of scientists in this field, especially in the "translational research" era.

5. Future directions

The next generation of SBS will need to perform significantly better than the actual synthetic bone grafts and compare or even

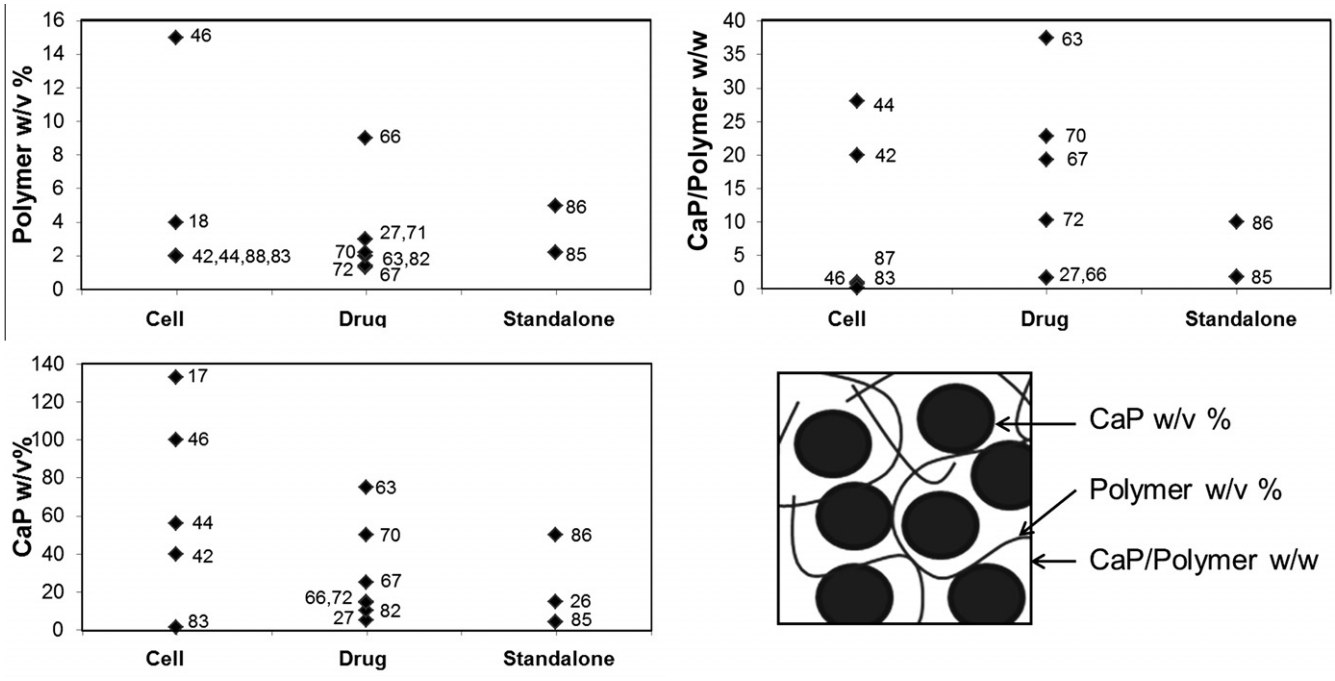

Fig. 3. Compilation of polymer content in % w/v, CaP% w/v and polymer/CaP ratio for the papers cited in this review, when data were available. Numbers indicate the bibliographic reference. Data sorted according to the use of composite alone (standalone), delivery of BMP-2 (drug) and cells (cell). The following references were included in the computation: [17, 18, 26, 27, 42, 44, 46, 63, 66, 67, 70–72, 82, 83, 85–88].

outperform autologous grafts. In this context, HG/CaP composites will probably have to embrace the role of drug delivery systems, but also play a role in the regeneration process combining intrinsic osteogenic property, ability to support cells and potentially gene therapeutics. Still, even before considering advanced new materials, there is a strong need for systematic studies designed solely to understand and optimize present composites in terms of composition, CaP particle and pore size, matrix rigidity, degradation profile and presence of biofunctionality. Research on ceramic particles has achieved extensive progress and finally commercial success. Importantly, from this review, the development of proper HGs carriers for SBS is more uncharted, and therefore open for further research.

Handling and injectability of HG/ceramic particle composites still present issues. New matrices that could significantly improve the composites include: HGs with high amplitude shear-thinning properties and fast reformation of the network [95]; multi-responsive HGs [96], thermoresponsive HGs with enhanced temperature–induced sol–gel transition [97]; interpenetrating networks [98]; inclusion of nanofibers into the HG matrix [65,99]; rosette nanotubes [100]; and inclusion of anionic phosphate groups within the HG polymer [101]. Another possible direction in research is the formulation of composites in water-free polymeric carriers [102].

Further directions for research in the area of HG/CaP composites for bone healing may involve the association of the composites with drugs and GFs different from BMP. Among them, small interfering ribonucleic acids [103] and associations of multiple factors playing a role in bone regeneration [84] are significant candidates. Moreover, in order to satisfy the growing demand of products tailored for specific therapeutic applications, future research should explore the association with antibiotics, anti-osteoporotic, anti-tumor and analgesic drugs [57,59,104].

The next generation of smart HGs for composites will confidently support the osteoconductive and osteoinductive potential of the CaP. Mata et al. [105] and Amosi et al. [86], for instance, reported peptidic HG scaffolds with the ability to act as nuclei for mineral formation and control the availability and release of calcium ions. Targeted and controlled degradation of the HG matrix in HG/CaP is also likely to be of profound importance for composite bone grafts. Indeed, when matrix metalloproteinase sensitive peptides were incorporated into a hyaluronan HG and combined with BMP-2, increased alkaline phosphatase and osteopontin levels were detected in vitro, and new bone formation was observed in a rat calvarial defect model [106]. This suggests that an improved HG design for SBSs could be a matrix degraded upon the presence of biological factors expressed during the early bone healing process.

The mechanical properties of the composites and relative response of the damaged biological tissues are also important features of bone repair. These aspects have not yet been fully considered in HG/CaP composites, as demonstrated by the lack of reported mechanical evaluation. The inherent difficulty in modulating the mechanics of HG/CaP independently of the composition makes it relatively complicated to study this effect. Moreover, most of the bone graft substitutes aim to speed-up the healing of bones, which are formed through an endochondral mechanism (e.g., long bone, mandible, spine vertebrae). Thus, developing very strong HG/CaP composites, especially for a cell therapy approach, may be less desirable than weaker matrices directing bone healing toward an endochondral pathway [89].

Of course, all these promises need to be confirmed upon solid foundations of experimental evidence. The use of fibrin sealants in bone healing gives a clear example of a carrier that features good potential (good tolerability, initial stability, mechanical resistance, inherent cell attachment sites, positive role played in vascularization, biodegradability), but efficacy is still controversial 35 years after the first positive evidence [11].

Acknowledgments

Dr. Marianna Peroglio and Ryan Seelbach are gratefully acknowledged for the inspiring revision of the manuscript and the literature research, respectively. This work received funding from the European Union's 7th Framework Program under Grant Agreement Nos. NMP4-L-2010-262948 (Biodesign) and NMP3-SL-2010-245993 (GAMBA).

Appendix A. Figures with essential color discrimination

Certain figure in this article, particularly Fig. 2 is difficult to interpret in black and white. The full color images can be found in the on-line version, at doi: http://dx.doi.org/10.1016/j.actbio.2012.11.022.

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