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Understanding calcification in bone regeneration through a
synthetic biomineralization-based approach

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Bioengineering

by

Ameya Phadke

Committee in charge:

Professor Shyni Varghese, Chair
Professor Ratneshwar Lal
Professor Koichi Masuda
Professor Paul Price
Professor Robert Sah

2012

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University of California, San Diego

2012

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Chapter 5 is currently being prepared as a manuscript for publication, titled “Synthetic biomineralized matrices as osteoinductive bone grafts for posterolateral fusion: an *in vivo* study”

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‘*’ indicates equal contribution from both authors

Abstract of the Dissertation

Understanding calcification in bone regeneration through a synthetic biomineralization-
based approach

by

Ameya Phadke

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2012

Professor Shyni Varghese, Chair

Bone is one of the most transplanted tissues, with over 500,000 bone grafting procedures carried out annually in the United States. While autografts, allografts and recombinant protein-based devices are widely used in these procedures, they suffer from

numerous shortcomings such as cost, donor site morbidity, batch-to-batch variation and potential risks & side effects. This thesis demonstrates the development of biomineralized synthetic grafts and investigates their efficacy by probing their ability to induce osteoinduction *in vitro* and *in vivo*.

Briefly, Chapter 1 describes the current state of the field in the use of calcium phosphate-based biomaterials for bone regeneration, as well as current progress in the development of mineral-polymer composites. Chapter 2 describes the development of a novel mineralization process to synthesize biomimetic mineral-synthetic hydrogel composites and utilizes this process to probe the effect of matrix hydrophobicity on the templated mineralization of these substrates. This conclusively demonstrates a non-monotonic dependence of matrix hydrophobicity and pendant side chain length on the templated mineralization of synthetic materials, and further demonstrates that hydrogels containing N-acryloyl 6-aminocaproic acid-based moieties as optimally suited for templated mineralization. Chapter 3 then investigates the osteoinductivity of the biomineralized A6ACA hydrogels *in vitro*. Indeed, these matrices were found to promote the osteogenic differentiation of human mesenchymal stem cells even in the absence of soluble osteogenic supplements, suggesting their ability to promote osteoinduction. Chapter 4 then proceeds to investigate two potential pore structures for use in porous synthetic grafts through their ability to support osteogenic differentiation of hMSCs *in vitro*. A pore structure consisting of isotropic, cellular pores was found to support osteogenic differentiation to a greater extent than a pore structure consisting of lamellar, oriented pores. Furthermore upon mineralization, these matrices were found to promote

extensive vascularization and ectopic bone formation upon subcutaneous implantation in nude rats both with and without pre-seeded hMSCs prior to implantation, thereby providing evidence of their osteoinductive potential. Chapter 5 then proceeds to evaluate these grafts in a rodent posterolateral fusion model; the biomineralized grafts were found to promote spinal fusion to a similar extent, both with and without addition of bone marrow flush and to a significantly greater extent than sham surgery. Finally, Chapter 6 demonstrates the tuning of interfacial properties for eliciting matrix-matrix interactions for the creation of hydrogels capable of undergoing rapid, and reversible self-healing via cross-interfacial hydrogen bonding.

CHAPTER 1: Introduction

1.1. Biomineralization

Biomineralization refers to the process of controlled deposition of inorganic elements in the form of mineral phases in by biological organisms. Examples of biomineralization include the mineralization of bone and teeth in vertebrates, the formation of calcified shells in mollusks as well as the formation of mineral deposits by bacteria (Mann, 2001). This typically involves the organisms producing an organic matrix capable of inducing the growth of specific minerals in a controlled manner. Biomineralization can serve a variety of functions, from structural support, protection, storage of inorganic cations and anions and even utilizing the response of the minerals to magnetic fields for navigation. This chapter will discuss current developments regarding the knowledge of biomineralization pertaining to the development and regeneration of bone in mammals and its capitulation through synthetic systems.

1.2. Bone

Bone is a rigid system of organs that provide structural support to the body, and is composed of hard connective tissue, as well as soft tissue components such as an outer layer (referred to as the periosteum) and bone marrow, which contains mesenchymal progenitors, fat, hematopoietic progenitors and an assortment of growth factors. Bone possesses a highly hierarchal architecture; an understanding of this organization is necessary from both, a compositional and cellular perspective in order to gain insights

into the processes of biomineralization pertaining to bone regeneration. Figure 1.1. summarizes the hierarchial processes involved in the biomineralization of bone.

1.2.1. Composition of bone

At the molecular level, bone is unique due to its composite structure consisting of organic and inorganic components. The inorganic component is a calcium phosphate-based mineralized phase. While this phase is somewhat similar to hydroxyapatite in crystalline structure, studies have yielded significant differences between bone mineral and hydroxyapatite, especially via the substitution of PO_4^{3-} groups and to a lesser extent, OH groups, with CO_3^{2-} groups (Wopenka and Pasteris, 2005). At the microscopic level, these minerals appear to form lamellar plates within the interfibrillar spaces on the collagen fibrils within bone. The presence of these plates of mineral imbues the bone with exceptional mechanical strength, allowing it to resist large forces and thereby making it ideal to serve as structural support. The presence of the carbonate groups also makes bone mineral more amenable to dissolution, as carbonated apatite has a significantly higher solubility product than hydroxyapatite (Tang *et al.*, 2003); the role of dissolution and reprecipitation of the mineralized phase will be discussed in subsequent sections of this chapter. The mineralized phase also serves as a reservoir for Ca^{2+} and PO_4^{3-} , as these ions are critical in a number of physiological processes.

The organic component (known as osteoid) is composed predominantly of type I collagen, proteoglycans and non-collagenous proteins, and increases the toughness of the bone. Non-collagenous proteins found in bone include osteocalcin, bone sialoprotein, osteopontin, bone morphogenetic protein, and phosphorylated glycoproteins such as

osteonectin (Termine, 1988). These proteins play an important role in the formation and maintenance of bone tissue, as well as providing a number of instructive signals critical to its function, particularly pertaining to its mineralization. Specifically, immunohistochemical analysis of the localization of these proteins has yielded insights into their function. Osteopontin and bone sialoprotein have been observed to typically lie ahead of the mineralization front and as a result, could potentially play a role in the initiation of the formation of the mineralized phase. Osteocalcin and osteonectin have been observed in mature mineralized tissue, and could thus play a role in controlling the growth of the crystalline phase after initial formation (Roach, 1994). The role of osteocalcin is particularly intriguing, given that it possesses specific carboxyglutamate residues that enhance the affinity for binding to Ca^{2+} ions and align with the lattice spacing of Ca^{2+} in the xy plane of hydroxyapatite, suggesting its excellent affinity for binding to apatites (Hauschka and Wians, 2005; Price, 1989).

1.2.2. Cellular components of bone

Bone contains several cell types: osteoblasts, osteocytes and osteoclasts (Nijweide *et al.*, 1986). These cells each play a vital role in the formation, repair, remodeling and turnover of bone tissue. Osteoblasts are mononucleated, cuboidal cells arising from the differentiation of mesenchymal stem cells into the osteogenic lineage; these cells are responsible for the production of the non-mineralized osteoid which then undergoes mineralization to form mature bone matrix. The differentiation of mesenchymal stem cells into osteoblasts is marked by several events at the gene expression level (Granchi *et al.*, 2010). Specifically, the early differentiation of mesenchymal stem cells into

osteoprogenitors is marked by an increase in alkaline phosphatase activity and the expression of the osteogenic master regulating transcription factor Runx2. The subsequent differentiation of osteoprogenitors into mature osteoblasts is then marked by a decrease in expression of Runx2 and alkaline phosphatase, as well as subsequent expression of the osteogenic transcription factor osterix and genes encoding for osteoblast-secreted products such as osteocalcin, type I collagen, bone sialoprotein and osteopontin.

Osteoclasts are multinucleated cells that are hematopoietic in origin, and are responsible for the resorption of bone matrix. These cells are marked by the expression of tartrate-resistant acid phosphatases (TRAPs) and a ruffled cytoplasm, which allows for the generation of an isolated highly acidic micro-environment which allows for the dissolution of the mineralized phase and the degradation of the osteoid (Vaes, 1988). The released Ca^{2+} and PO_4^{3-} ions are then endocytosed by the osteoclasts and released into the extracellular fluid, making them available for various physiological processes. The antagonistic activity of osteoblasts and osteoclasts is essential for Wolff's law which describes the ability of bone to undergo remodeling in response to changes in mechanical loading (Frost, 1998). Under increased loading, there is an increase in osteoblastic activity and a corresponding decrease in osteoclastic activity, leading to a net increase in bone deposition. Under decreased mechanical loading, there is a net decrease in osteoblastic activity and an increase in osteoclastic activity leading to net bone resorption.

Osteocytes are terminally differentiated osteoblasts that have become entrapped within bone (Tate *et al.*, 2004). Unlike osteoblasts, osteocytes have extremely limited synthetic activity; rather, they have demonstrated a limited ability to resorb bone, referred to as ‘osteocytic osteolysis’. Recent studies however have established that osteocytes function as exquisitely sensitive mechanosensors and mediate osteoblastic and osteoclastic activity upon sensing of mechanical stimuli (Jacobs *et al.*, 2010). This work however, will focus primarily on the modulation of osteoblastic activity and differentiation.

1.3. Bone grafting

Despite bone’s excellent ability to repair damage, several conditions often necessitate the transplant of healthy bone into the damaged site to facilitate adequate bone formation. In fact, it is estimated that over 500,000 bone grafting procedures are performed annually (Faour *et al.*, 2011). A variety of procedures require the use of bone grafts; these include spinal fusion procedures and the repair of large defects in the bone, arising from trauma or from excision of bone due to tumor invasion or infection. Approximately half of the bone grafting procedures are performed for spinal fusion, typically performed for the mitigation of debilitating back pain arising from damage to the intervertebral disc, spondylolisthes and other spinal disorders which necessitate fusion of vertebrae. While the nature of fusion varies from procedure to procedure, spinal fusions typically involve placement of a graft between the adjacent vertebrae to be used, either between the vertebral bodies (referred to as interbody fusion) or between the transverse processes on either side of the desired fusion level (referred to as posterolateral

fusion). The graft then encourages both, the in-growth of native bone at the fusion level as well as formation of new bone within the fusion region, thus leading to the formation of a bony fusion mass between the vertebrae and thus leading to their fusion.

1.3.1. Current bone grafting strategies

In order for materials to promote bone regeneration, they must be osteoconductive, osteoinductive and osteogenic (Giannoudis *et al.*, 2005).

Osteoconduction refers to the facilitated in-growth of native bone tissue at the implant site and is predominantly governed by the graft-guided infiltration of osteoblasts and osteoclasts into the implant region, and their subsequent production of mature bone at the implant periphery. This is essential for the integration of the implant and newly formed bone with the native bone tissue. Osteoinduction refers to the ability of the material to recruit mesenchymal progenitors and subsequently stimulate their differentiation into an osteoblastic lineage, allowing for deposition of new bone matrix within the implant. Finally, osteogenesis refers to the subsequent formation of *de novo* mature bone within the implant. Currently, bone grafting typically utilizes autografts, allografts, xenografts or synthetic substitutes. Autografts refer to bone sourced from the same patient, typically from the iliac crest. This is currently considered a gold standard and is typically associated with excellent clinical outcomes. However, the autografting procedure is associated with numerous shortcomings, such as donor site morbidity and the highly invasive nature of the technique. These are also typically associated with significant post-operative pain from the grafting procedure and can lead to chronic discomfort for the patient. Additionally, specific patient subpopulations can preclude the use of autografts

due to the inherently poor bone forming ability; these include elderly women, particularly those suffering from osteoporosis and smokers, as these criteria have been typically associated with less than ideal clinical outcomes (Ludwig *et al.*, 2000). Allografts refer to bone from another human patient, often from a cadaveric source. These can be implanted as is in the form of viable bone, or post-processed to remove specific components such as the mineralized phase for ease of handling characteristics as seen with demineralized bone matrix (DBM). Allografts pose the risk of immunorejection and infection transmission and often have to undergo extensive characterization and patient-matching, thereby requiring the maintenance of extensive tissue banks. Moreover, allografts are highly susceptible to batch-to-batch variability, arising from differences in the osteogenic capacity of bone sourced from different donors. These factors substantially increase the cost and risk associated with their use.

Given the aforementioned issues arising from the use of autografts and allografts, recent times have witnessed the development of synthetic grafting strategies. These can broadly be classified into (a) ceramic-based materials such as calcium phosphates and bioactive glasses and (b) growth factor-based strategies. Growth factor-based strategies typically utilize recombinant growth factors such as bone morphogenetic proteins (BMPs)- specifically, rhBMP-2 and rhBMP-7 have been approved for clinical applications requiring osteoinduction (Cheng *et al.*, 2003). These growth factors are delivered using a resorbable carrier matrix such as collagen sponges or resorbable polymeric materials that allow for their temporary immobilization and delivery to the damage site. INFUSE (Medtronic Inc) is an example of an FDA-approved growth factor-

based commercial product and utilizes recombinant BMP-2, soaked in a collagen sponge. Indeed, these materials have demonstrated an osteoinductive capacity at least comparable to autografts and have even surpassed autograft performance in several studies (Burkus *et al.*, 2003; Dimar *et al.*, 2006). Despite their obvious powerful osteoinductivity, recombinant protein-based grafting strategies face some important issues in their applications. These proteins typically have a relatively short half-life due to dispersion as well as degradation and as a result, require relatively large doses for therapeutic efficacy—approximately three orders of magnitude higher than their native concentration (Issa *et al.*, 2008; Termaat *et al.*, 2005). High doses of these proteins can result in harmful side effects such as ectopic bone formation, respiratory side effects, reproductive side effects, increased risk of cancer and osteolysis (Carragee and Weiner, 2011; Kang, 2011). Due to the relatively high dosage, coupled with the high costs associated with the production of these factors and their batch-to-batch variability, the costs of BMP-based grafting strategies are also extremely high, with a single dose costing approximately \$5,000 (Capo *et al.*, 2011). Due to these factors, the use of recombinant growth factors is thus advisable only in cases wherein patients exhibit a dramatically reduced bone-forming capacity, such as in heavy smokers and individuals with severe cases of osteoporosis.

1.3.2. Calcium phosphate-based bone graft substitutes

Due to the aforementioned issues with growth factor-based strategies, recent years have witnessed the development of a number of synthetic materials for use as bone grafts. Synthetic materials do not suffer from the batch-to-batch variability associated with autografts, allografts and to a lesser extent, the recombinant growth factor-based

strategies. Moreover, these materials are associated with a much lower cost of production. As a result, the use of bone graft substitutes based on synthetic materials is highly promising. Given the mineralized structure of bone, the use of ceramic, crystalline materials is an obvious choice for synthetic bone grafts. Indeed, ceramics such as bioactive glasses as well as calcium phosphates such as hydroxyapatite and β -tricalcium phosphate (β -TCP) have a well-demonstrated ability to bond to bone (LeGeros, 2002). Studies have revealed that these materials, upon exposure to physiological fluid *in vivo*, form a surface layer of biological apatite that is similar to bone mineral; this effectively stimulates the infiltration of these materials by native osteoblasts, allowing for effective osseointegration of the materials (Barradas *et al.*, 2011).

Intriguingly, synthetic materials have also demonstrated some degree of osteoinductivity *in vitro* and *in vivo* (Barradas *et al.*, 2011). A number of calcium phosphates and bioactive glasses have demonstrated the ability to promote the osteogenic differentiation of progenitor cells *in vitro* even without the presence of exogenously added osteogenic supplements in the culture medium (Cameron *et al.*, 2012; Müller *et al.*, 2008). This osteostimulatory effect has been attributed to a variety of mechanisms such as the release of inorganic ions, topographical and microstructural cues and selective adsorption of osteoinductive growth factors from culture medium and activation of pro-osteogenic adhesion molecules (Barradas *et al.*, 2011). In addition to such ceramic-based materials, osteogenic differentiation of hMSCs has also been demonstrated on synthetic polymeric materials via physicochemical cues such as matrix stiffness (Engler *et al.*,

2006), surface chemistry (Benoit *et al.*, 2008), matrix hydrophobicity (Ayala *et al.*, 2011), topography (Dalby *et al.*, 2007) and cell adhesion ligands (Shin *et al.*, 2005).

Osteoinduction *in vivo* however, has typically been restricted to calcium phosphates and that too, in specific animal models. Evaluation of the osteoinductive capacity of materials involves their implantation at a non-osseous site such as in a subcutaneous or intramuscular location, and subsequently evaluating the formation of bone mediated by this material. This was first demonstrated by Urist (Urist, 1965), who found that demineralized bone matrix when implanted subcutaneously, induced neo-bone formation in rats. Indeed, vast number of subsequent studies have demonstrated the ability of biologic molecules to induce bone formation, as described earlier. Synthetic materials were then subsequently demonstrated in several studies to induce ectopic bone formation in mammals; specifically, Selye *et al.* found that glass diaphragms induced ectopic bone formation in rats upon subcutaneous implantation (Selye *et al.*, 1960) and Winter demonstrated reproducible formation of bone in a polymeric poly(2-hydroxy ethylmethacrylate)-based sponge upon subcutaneous implantation in pigs (Winter, 1970). Winter also noted that the sponge underwent *in vivo* calcification prior to the formation of ectopic bone, suggesting that the presence of a mineralized phase promoted the formation of subcutaneous bone within the sponge. This was in agreement with previous studies, which found calcified soft tissues such tendons induced ectopic bone formation *in vivo*, but not without pre-calcification prior to their implantation (Habibovic and de Groot, 2007). These studies indicate that in addition to biologic molecules such as bone

morphogenetic proteins, calcium phosphate-based materials could possess intrinsic osteoinductivity.

Subsequent studies have exhaustively evaluated the ability of calcium phosphate-based porous materials to induce ectopic bone formation. Indeed, a variety of calcium phosphate-based materials such as β -TCP, hydroxyapatite and biphasic calcium phosphate (consisting of a combination of β -TCP and hydroxyapatite) and octacalcium phosphate have been demonstrated to induce bone formation in soft tissues, particularly in large animal models, although this has rarely been noted in small animals such as rodents (Habibovic *et al.*, 2004; LeGeros, 2002; Yuan *et al.*, 1998). Among these materials, β -TCP, BCP and OCP have typically shown superior osteoinductivity when compared to hydroxyapatite. One of the potential mechanisms suggested to be underlying this phenomenon is the dynamic dissolution and precipitation of the mineralized phase. It has been postulated that the dissolution of calcium phosphates *in vivo*, followed by their reprecipitation into a biological apatite-like layer could lead to increased local concentrations of Ca^{2+} and PO_4^{3-} ions, as well as selective sequestration of osteoinductive growth factors such as BMPs in the implant (LeGeros, 2002). Indeed, it has been conclusively demonstrated that exposure to increased Ca^{2+} and PO_4^{3-} concentrations is sufficient to drive the osteogenic differentiation of multipotent progenitors without any other stimuli (Chai *et al.*, 2010; Chai *et al.*, 2011). Moreover, while studies have demonstrated that BMPs do have some preferential affinity to bind to calcium phosphate substrates (Urist *et al.*, 1984), it is also possible that they bind to the mineralized matrix

via interactions with fetuins which have a strong affinity to bind to calcium phosphate nuclei and also possess strong affinity to BMPs (Binkert *et al.*, 1999).

Along with the chemical composition, microstructural topography of calcium phosphate based materials has been shown to play a significant role in calcium phosphate-induced osteogenesis. Yuan *et al.* demonstrated that biphasic calcium phosphates with an identical chemical composition but sintered at different temperatures exhibited differential osteoinductive capacities *in vivo* (Yuan *et al.*, 2010). This finding agrees with recent findings that suggest that topographical cues can stimulate osteogenic differentiation of hMSCs even without the presence of exogenous osteogenic stimuli in the medium (Dalby *et al.*, 2007). Moreover, other studies have also demonstrated that microtopography and microporosity of calcium phosphates play a significant role in ectopic bone induction (Yuan *et al.*, 1999). These properties can influence dissolution-reprecipitation of the mineralized phase, as well as growth factor adsorption; based on previous reports it is likely that these two phenomena govern the osteoinductivity of calcium phosphates *in vivo*.

1.3.3. Synthetic composite matrices

As mentioned previously, calcium phosphate biomaterials are highly promising as materials for bone regeneration. However, these materials suffer from a number of shortcomings- in particular, the biological apatite surface layer associated with their bioactivity is easily delaminated, primarily due to the lack of strong adhesion between the native ceramic material and the mineralized layer (Song *et al.*, 2003). Moreover, these materials do not effectively mimic the composite structure of bone; in particular, calcium

phosphate ceramics are typically highly crystalline, as compared to the semicrystalline nature of bone mineral (Gryn timer, 1976). Additionally, the organic substrate could also serve as a catchment reservoir for osteogenic proteins such as BMPs selectively adsorbed by minerals from body fluids, potentially leading to a sustained increased concentration of these factors within the material. These materials are typically more flexible and possess improved flexural strength when compared to ceramic scaffolds which are typically highly brittle. From the standpoint of ease of use, this also leads to improved handling characteristics and thereby makes their practical application more promising.

Several studies have created organic/inorganic composites as nanocomposites through dispersion of calcium phosphate particles in an organic phase, such as collagen, or through the coating of an organic substrate with nano-sized particles of calcium phosphate. These studies have demonstrated that such composites can promote osteogenic differentiation *in vitro* (McCullen *et al.*, 2009; Polini *et al.*, 2011), bone regeneration *in vivo* both with and without exogenously added cells (Cowan *et al.*, 2004; Wang *et al.*, 2007) and even osteoinduction upon ectopic implantation in subcutaneous or intramuscular sites (Pek *et al.*, 2008; Seyedjafari *et al.*, 2010). Several commercial products with this composite structure are also currently available; some examples include Formagraft (NuVasive) and Collagraft (Zimmer), both of which are composites of crystalline calcium phosphates and type I collagen and have demonstrated some success in bone regeneration and spine fusion *in vivo* (Vaccaro *et al.*, 2002; Walsh *et al.*, 2009)

1.3.4. Biomineralization of organic matrices

While composite materials formed by physical mixing of minerals in an organic phase have indeed demonstrated increased bone healing capacity when compared to minerals alone, they are also less-than-ideal mimics of bone. Bone is formed through the growth of the mineralized phase within the osteoid and not from physical mixing of the organic and inorganic phases as is the case with these materials; they thus mimic bone from a compositional standpoint in that they consist of an organic phase and a mineralized phase; they do not however, mimic bone from a structural perspective at the molecular level. Indeed, the dynamic dissolution-precipitation widely attributed to be underlying the osteostimulatory effect of calcium phosphates would likely be more prevalent in the semicrystalline minerals in bone as opposed to the highly crystalline minerals used in the synthesis of these composites, as the former is more amenable to dissolution on account of their reduced crystallinity .

This can be addressed through another method for the synthesis of mineralized composites, wherein mineralized phase is grown on an organic substrate in a templated fashion, rather than their physical mixing with the organic phase- in other words, the organic substrate directs the growth of the mineral rather than merely serving as a structural medium for the formation of the mineralized phase (Kretlow and Mikos, 2007). This is similar to the mechanisms underlying the mineralization of osteoid, wherein several non-collagenous proteins such as bone sialoprotein, osteopontin, osteonectin and osteocalcin are believed to initiate and control the growth of the mineralized calcium phosphate phase on the collagen fibrils. A popular method for the mineralization of these

organic matrices is their incubation with solutions containing Ca^{2+} and PO_4^{3-} ions. One solution which is particularly widely used is referred to as simulated body fluid, or SBF (Oyane *et al.*, 2003); this solution mimics the ionic composition of plasma and maintains a physiological pH of 7.4, allowing for the growth of a carbonated apatite that bears similarity to biological bone mineral observed *in vivo* (Murphy and Mooney, 2002).

A prevalent method for mineralization of synthetic matrices involves incorporation of moieties that can induce calcium phosphate formation. Several non-collagenous proteins have been determined to be rich in anionic residues, specifically those from glutamic acid and aspartic acid (Hunter and Goldberg, 1993; Hunter and Goldberg, 1994; Hunter *et al.*, 1996). These residues contain pendant charged groups which can then bind Ca^{2+} and subsequently initiate the nucleation of the mineralized phase. There is a strong chemical bond between the organic proteins and the inorganic mineral, leading to an extremely strong osteoid-mineral interface. Inspired by this, a number of studies have demonstrated that synthetic matrices can be mineralized through the incorporation of negatively charged functional groups such as carboxyl and phosphate groups, and positively charged imine groups (Ball *et al.*, 2006; Huang *et al.*, 2010; Kawashita *et al.*, 2003; Suzuki *et al.*, 2006). In addition to directly serving as nucleation points during mineralization, charged functional groups can also promote the binding of charged proteins (Kato *et al.*, 1995; Shelton *et al.*, 1988); given that numerous studies have demonstrated the ability of proteins to promote the nucleation of calcium phosphate in solution (Daculsi *et al.*, 1999; Rohanizadeh *et al.*, 1998; Zainuddin *et al.*, 2006), it is highly likely that these adsorbed proteins can promote the mineralization of the

polymeric substrates, thereby yielding an indirect method through which charged functional groups can be utilized for the mineralization of synthetic materials. Indeed, SBF-containing albumin has been demonstrated to form a bone-like mineral layer on poly(2-hydroxyethylmethacrylate) substrates (Zainuddin *et al.*, 2006); this was likely due to the adsorption of albumin on the surface, followed by the albumin-mediated nucleation of the mineral layer on the material surface. In addition to merely mimicking the role of individual functional groups in biomineralization, several studies have also illustrated the utility of mimicking the secondary structure of proteins associated with biomineralization *in vivo*. It has been demonstrated that the β -sheet rich secondary structure of bone matrix proteins play a critical role in their ability to bind to and nucleate apatite (Addadi and Weiner, 1985). Inspired by this phenomenon, studies have created self-assembling peptide-based systems that mimic this structure and are able to undergo biomineralization (Hartgerink *et al.*, 2001; Segman-Magidovich *et al.*, 2008). Along with small functional groups, a limited number of studies have also demonstrated that incorporation of large molecules into synthetic materials can also facilitate their mineralization. For example, inspired by the well-established role of alkaline phosphatases in bone mineralization *in vivo*, Filmon *et al.* demonstrated that poly (2-hydroxyethyl methacrylate) substrates functionalized with alkaline phosphatase underwent mineralization when immersed in simulated body fluid supplemented with β -glycerophosphate (Filmon *et al.*, 2000). The cleavage of β -glycerophosphate by the alkaline phosphatase likely led to a local increase in phosphate ions and the subsequent deposition of the mineralized phase on the polymeric substrate on account of supersaturation.

Synthesis of organic/inorganic composite materials through templated mineralization has demonstrated many advantages over physical mixing of calcium phosphate particles in an organic phase. First and foremost, these materials more effectively mimic bone at the molecular and compositional level particularly through the presence of strong chemical interactions between the mineralized and organic phases, as the process of templated mineralization is similar to the mechanisms underlying bone mineralization. As in bone, the mineralized phase for synthetic composites is semicrystalline, as opposed to the highly crystalline nature of the mineralized phase obtained when synthetic calcium phosphate particles are physically dispersed in an organic phase. The semicrystalline nature of the mineral phase is also more amenable to dynamic dissolution and reprecipitation which has been implicated as a possible mechanism underlying the osteoinductivity of calcium phosphates. Studies have indeed shown that matrices produced by templated mineralization show a superior osteostimulatory effect when compared to composite matrices produced by mixing of mineral particles in the organic phase (Osathanon *et al.*, 2008). Finally, the presence of chemical linkage between the mineralized and organic phases leads to a strong mineral-substrate interface, thereby preventing separation or delamination of these phases (Song *et al.*, 2003).

Yet another bio-inspired technique for the mineralization of organic substrates involves using fetuin to mediate the formation of a calcium phosphate phase. Fetuins are extremely abundant in serum and other body fluids, and possess a striking affinity for calcium phosphate nuclei (Price *et al.*, 2003). Given that body fluids are typically

supersaturated with respect to calcium phosphate, these nuclei can spontaneously form in suspension. However, fetuins then bind to these nuclei and further prevent their growth the subsequent formation of mature crystallites, preventing mineralization of tissues under normal circumstances. In the case of osteoid, the densely packed matrix prevents diffusion of fetuins into the interior via size exclusion, on account of their large size (~50-60 kDa). Ca^{2+} and PO_4^{3-} ions on the other hand are much smaller in size and thus readily penetrate the osteoid accumulating between the collagen fibrils in the matrix; they then reach supersaturation and undergo nucleation into calcium phosphates. However, the lack of fetuins in the interfibrillar space allows these nuclei to mature into apatite crystallites and proceed to form mineralized plates in the interfibrillar space, as well as inside the fibrils (Price *et al.*, 2009). Acidic groups in the collagen and non-collagenous proteins can then direct the geometry and phase of this mineralized component into a semicrystalline apatite-like structure. This mechanism has been utilized to re-mineralize demineralized bone (Price *et al.*, 2004) and could thus also be harnessed for the mineralization of polymeric hydrogels, through networks that would allow Ca^{2+} and PO_4^{3-} ions but not fetuins to infiltrate the gel interior via size exclusion.

While numerous studies have investigated the *in vitro* and *in vivo* response to organic/inorganic composites prepared by physical mixing as detailed earlier, there are a limited number of studies which have investigated the effects of biomineralized materials on osteogenesis. Of these, a majority of reports have demonstrated that such materials are at the very least, able to support osteogenic differentiation and osteoblast maturation (Hild *et al.*, 2011; Liu *et al.*, 2009); a limited number of studies however, have

demonstrated that the presence of a calcium phosphate mineral film can inhibit the osteogenic differentiation of progenitor cells, especially in the presence of osteogenic culture medium (Murphy *et al.*, 2005). This discrepancy could possibly be due to the different phases of calcium phosphates formed in different studies. Differences in phases could lead to differences in topography, growth factor adsorption as well as a differential rate of dissolution. Studies have demonstrated that while increases in Ca^{2+} and PO_4^{3-} concentrations can stimulate osteogenic differentiation of progenitor cells, there is a relatively narrow range over which they can do so; excessively high Ca^{2+} and PO_4^{3-} concentrations can lead to cell death and even inhibit osteogenic differentiation (Liu *et al.*, 2009)- this could be the reason underlying the increased cell death and reduced osteogenic differentiation previously reported on amorphous calcium phosphate substrates as compared to crystalline calcium phosphates (ter Brugge *et al.*, 2002). Organic substrates and mineralization conditions that favor the formation of phases that are either highly crystalline or lack bioactivity could lead to insufficient dissolution and reprecipitation of the mineralized phase, thus decreasing their resultant osteogenic response. On the other hand, organic substrates and mineralization conditions that favor the formation of phases that are excessively amorphous in nature could lead to excessive dissolution and reprecipitation, potentially leading to a cytotoxic environment that can impede osteogenesis. It is thus apparent that the underlying organic substrate and the mineralization procedure can play a critical role in determining the osteoinductivity of the biomineralized phase. The design of biomineralized composites thus requires the systematic engineering of an organic substrate and mineralization conditions that are able to generate a biomineralized phase capable of promoting bone formation.

In summary, synthetic materials composed of an organic phase and an inorganic, biomineralized phase that mimic the composite structure of bone are promising alternatives to autologous bone grafts, allografts and recombinant growth factor based therapies. By mimicking the native biomineralized structure of bone, these substrates can provide the physicochemical cues necessary for the recruitment of host progenitors, their directed differentiation to a bone-specific phenotype and the subsequent formation of neo-bone tissue. This work describes a series of studies detailing the development of such composites and their *in vitro* and *in vivo* evaluation as synthetic bone grafts, as defined by their ability to promote osteogenic differentiation of stem cells *in vitro*, and subsequently ectopic bone induction and posterolateral fusion *in vivo*.

1.4. Figures

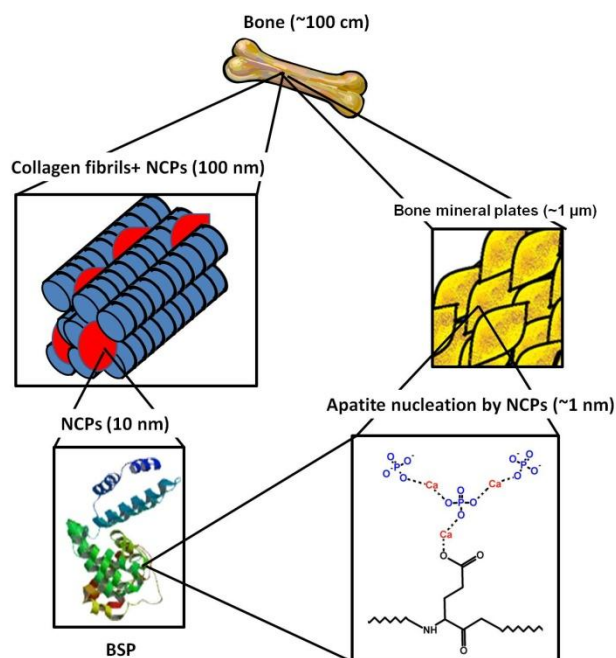


Figure 1.1. Hierarchical organization of biomineralization processes in bone

Bone consists of two phases: an organic proteinaceous phase called osteoid that consists of collagen type I and NCPs (non-collagenous proteins) and plates of calcium phosphate-based bone mineral. At the protein level, the NCPs (which direct the growth of the mineralized phase) consist of proteins such as bone sialoprotein (BSP), which contain pendant carboxyl groups that, at the molecular level, can bind to and nucleate calcium phosphates.

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CHAPTER 2: Templated Mineralization of Synthetic Hydrogels for Bone-like Composite Materials: Effect of Matrix Hydrophobicity

2.1. Abstract

Bone-mimetic mineral/polymer composite materials have several applications ranging from artificial bone grafts to scaffolds for bone tissue engineering; templated mineralization is an effective approach to fabricate such composites. In this study, we synthesized bone-like composites using synthetic hydrogels having pendant side chains terminating with carboxyl groups, as a template for mineralization. The role of matrix hydrophobicity on mineralization was examined using poly(ethylene glycol) hydrogels modified with varying lengths of anionic pendant side chains ($\text{CH}_2=\text{CHCONH}(\text{CH}_2)_n\text{COOH}$, where $n=1,3,5$, and 7). The ability of these hydrogels to undergo templated mineralization was found to be strongly dependent upon the length of the pendant side chain as evident from the extent of calcification and morphology of the minerals. Moreover, mineralized phases formed on the hydrogels were confirmed to resemble apatite-like structures. In addition to demonstrating the importance of material hydrophobicity as a design parameter for the development of bone-like synthetic materials, our study also provides a potential explanation for the *in vitro* differences between apatite-nucleating capacity of aspartate-rich osteopontin and glutamate-rich bone sialoprotein.

2.2. Introduction

Bone is a complex tissue with a composite extracellular matrix, consisting of an organic protein component (known as the osteoid) and an inorganic mineral phase, similar in structure to apatites such as hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) and dahllite (carbonated apatite) (Posner and Betts, 1975; Weiner and Traub, 1992; Weiner and Wagner, 1998). The osteoid on the other hand, consists predominantly of type I collagen along with other components frequently found in extracellular matrices and noncollagenous proteins such as osteocalcin, osteopontin, and bone sialoprotein (Weiner and Wagner, 1998). It is believed that the osteoid lends tensile strength while the mineral component provides rigidity to the tissue matrix. Mimicking this complex composite structure is essential for the development of synthetic bone graft materials that closely mimic natural bone. Such composite materials that mimic the structure of osseous and dental tissues also provide a tissue-specific microenvironment which can be harnessed to direct the differentiation of progenitor cells and stem cells into bone-specific cells (Engler *et al.*, 2009; Marino *et al.*, 2010; Yu *et al.*, 2009). Mineralized polymeric materials thus have immense applications both as bone grafts and as scaffolds in bone tissue engineering. Hence, there has been a recent surge in efforts to study formation of composite materials consisting of calcium phosphate minerals and substrates derived from both, synthetic and natural polymers (Kawashita *et al.*, 2003; Kretlow and Mikos, 2007; Osathanon *et al.*, 2008; Song *et al.*, 2003; Taguchi *et al.*, 1999).

Although the exact mechanism responsible for the *in vivo* mineralization of osseous tissue is unclear, a well-demonstrated and prominent factor in this phenomenon is the protein-mediated nucleation of apatite. Bone sialoprotein (BSP) is a glutamate-rich

protein believed to function as a nucleating agent *in vivo* through binding of calcium to the anionic glutamate residues (Goldberg *et al.*, 1996; Hunter and Goldberg, 1993). Similar studies have also illustrated the role of anionic residues of amelogenin in the mineralization of dental enamel matrix (Aichmayer *et al.*, 2009; Beniash *et al.*, 2005). Inspired by this mechanism, a popular method of inducing nucleation of calcium phosphate mineral phases is based on utilizing polymers with charged functional groups (Ball *et al.*, 2006; Kawashita *et al.*, 2003; Michel *et al.*, 2006; Ngankam *et al.*, 2000; Shkilnyy *et al.*, 2008; Suzuki *et al.*, 2006); thus initiating nucleation through binding of calcium or phosphate ions to anionic and cationic functional groups respectively. Another approach by Song *et al.* utilized the thermal decomposition of urea to facilitate formation of hydroxyapatite on poly(²hydroxyethyl methacrylate) matrices in a pH-mediated templating processes (Song *et al.*, 2003; Song *et al.*, 2005).

While the effect of chemical functionality on mineralization of polymeric matrices has been well studied, the effect of matrix hydrophobicity (independent of functional group) is yet to be studied. Previous studies by Sasaki *et al.* have demonstrated the effect of hydrophobicity of hydrogels containing carboxyl groups on Ca²⁺ binding (Sasaki *et al.*, 1998). This phenomenon may subsequently affect the capacity of hydrogels with varying hydrophobicity to nucleate calcium phosphate mineralized phases differently. The biomineralization process observed in nature during mineralization of bone tissue further emphasizes the potential role of matrix hydrophobicity on mineralization. For instance, despite being rich in anionic residues the aspartate-rich osteopontin (OPN) is less efficient at promoting mineralization than glutamate-rich bone sialoprotein (BSP) and under certain conditions does not nucleate apatites at all

(Goldberg *et al.*, 1996; Hunter and Goldberg, 1994; Hunter and Goldberg, 1993). The ability of peptides containing aspartate residues to suppress mineralization has also been demonstrated previously (Shiba and Minamisawa, 2007). One of the notable differences between the two proteins is that the glutamate residues (found in BSP) are more hydrophobic (longer alkyl chain) than aspartate residues (found in OPN). This clearly suggests that hydrophobicity of the templating matrix (independent of functionality) could be an important consideration in addition to functionality for biomineralization of synthetic matrices.

In this study, we evaluated the effect of matrix hydrophobicity on nucleation of apatite-like phases on a polymeric substrate. This was accomplished by synthesizing hydrogels with varying hydrophobicity by utilizing N-acryloyl amino acids with different number of methylene (CH₂) groups of the pendant side chain terminating with a carboxyl group (Ayala *et al.*, 2011; Badiger *et al.*, 1998; Varghese *et al.*, 2001a). These pendant side chains thus present Ca²⁺ binding-carboxyl groups, which serve as nucleation sites for the formation of crystalline calcium phosphates. Additionally, varying the number of CH₂ groups in increments of two groups at a time systematically allows for subtle changes in the matrix hydrophobicity without changing the terminal functional group. The ability of these matrices to undergo templated mineralization was evaluated using multiple approaches involving both protein-dependent and Ca²⁺-concentration dependent nucleation.

2.3. Results

N-acryloyl amino acid hydrogels with varying hydrophobicity were synthesized using PEGDA (MW: 6 kDa) as a crosslinker (Figure 2.1a-e). The effect of pendant side chain length on hydrophobicity (i.e. greater pendant side chain length increases matrix hydrophobicity) was confirmed through contact angle measurements (Figure 2.1f-g). The hydrogels were mineralized through three methods: (a) immersion in simulated body fluid (m-SBF), (b) immersion in m-SBF supplemented with fetal bovine serum (c) immersion in a metastable solution of 40 mM Ca^{2+} /24 mM HPO_4^{2-} . Figure 2.2a is a representation of the process of nucleation of calcium phosphate mineralized phases through Ca^{2+} binding to anionic surface moieties while Figure 2.2b represents mineralization through physisorption of proteins.

2.3.1. Mineralization with m-SBF

M-SBF was chosen for immersion because it closely mimics the ionic concentrations and pH typically observed in plasma (Oyane *et al.*, 2003). Upon soaking in simulated body fluid, faint white mineral deposits on A6ACA hydrogels were observed after approximately three weeks of immersion. While an analysis of Ca^{2+} content of these hydrogels using the calcium assay was not sufficiently sensitive to detect a difference in calcium content of the various hydrogels (data not shown), analysis with scanning electron microscopy (SEM) showed the formation of spherical particles consisting mainly of calcium phosphate for A6ACA hydrogels (Figure 2.3); deposits seen in other hydrogels (A2AGA, A4ABA, A8ACA) were found to be devoid of phosphate, as

evidenced by the elemental analysis by Energy Dispersion Spectra (EDS) (data not shown).

2.3.2. Mineralization with serum-supplemented m-SBF

Next, we supplemented the simulated body fluid with fetal bovine serum and evaluated the ability of serum proteins to promote mineralization. The ability of serum proteins such as fibronectin and albumin to nucleate apatite-like minerals under physiological conditions (Daculsi *et al.*, 1999; Zainuddin *et al.*, 2006) as well as the affinity for proteins such as albumin to charged polymeric substrates (Kato *et al.*, 1995) has been well established. Under these circumstances, the mineralization was faster than in the case of protein-free simulated body fluid, first appearing after 72 hours of immersion and peaking within 1 week of immersion despite an identical concentration of Ca^{2+} and HPO_4^{2-} in both solutions. Additionally, a pronounced and significant trend (One way ANOVA; $P < 0.001$) was observed wherein an increase in mineralization was observed concomitant an increase in side chain length as illustrated by increase in Ca^{2+} content, peaking for A6ACA, followed by a drastic decrease for A8ACA (Figure 2.4). These differences in rate and extent of mineralization suggest that adsorption of proteins from the serum onto the hydrogel surfaces played a pivotal role in the nucleation of minerals as per the mechanism in Figure 2.2b. The adsorption of proteins on the respective gels was confirmed through elution, followed by visualization by Coomassie blue staining (Figure 2.5).

Upon microscopic examination with SEM-EDS, A2AGA, A4ABA, A6ACA hydrogel surfaces exhibited continuous layers of calcium phosphate particles with

calcium/phosphate ratios of approximately 1.7, 1.7 and 1.6 respectively, as evidenced by elemental analysis through EDS; A8ACA hydrogels did not show any calcium phosphate deposits and showed minimal Ca^{2+} content (Figure 2.6). The similarity of the Ca/P ratios of the mineral phases on the hydrogels to that of synthetic hydroxyapatite (1.67), suggests the formation of an apatite-like phase. This was confirmed through XRD analyses (Figure 2.7), which indicated peaks corresponding to the diffraction spacing observed in hydroxyapatite (PDF-4-010-6312, based on PDF4+ ICDD database). This suggests the formation of semicrystalline hydroxyapatite on the hydrogels under physiological conditions. Other peaks observed include those corresponding to halite (NaCl) as well as unidentified peaks, presumably due to ordering within the gel due to the PEGDA oligomer (6 kDa), which is used as a crosslinker for the hydrogels (See Table 2.1 for detailed information on hydroxyapatite peaks observed in the XRD spectra of the hydrogels).

2.3.3. Mineralization with 40 mM Ca^{2+} /24 mM HPO_4^{2-}

We further investigated the effect of matrix hydrophobicity on templated mineralization independent of protein-surface interactions as per the mechanism outlined in Scheme 1A. Since the Ca^{2+} and HPO_4^{2-} concentrations in simulated body fluid were too low (2.5 mM for Ca^{2+} and 1.0 mM for HPO_4^{2-}) to promote extensive formation of a mineralized phase independent of proteins (as observed in experiments involving immersion in simulated body fluid), we developed a method utilizing a metastable solution containing elevated concentrations of Ca^{2+} (40 mM) and HPO_4^{2-} (24 mM). This method takes advantage of the high solubility of calcium phosphates under acidic

conditions. However, employing a low pH solution will result in protonation of the carboxyl functional groups on the hydrogels, thereby reducing the number of available Ca^{2+} -binding sites. By slowly raising the pH of this acidic solution through dropwise addition of 1 M Tris HCl (pH 7.5), the pH of the solution was raised to 5.2 while maintaining stability of the solution (without any observed turbidity for the duration of the 30 minute immersion). Significantly, this value was well above the pK_1 values (pertaining to the protonation/deprotonation of the terminal carboxyl group) of all the monomers, believed to be similar to those of the parent amino acids (See Table 2.2). This ensured that the protonation of the carboxyl groups did not compete with the binding of the pendant side chains to Ca^{2+} .

White mineral deposits were observed on hydrogels irrespective of their pendant side chain length within the first 30 minutes of immersion in the metastable solution. As observed with serum-supplemented SBF, calcium content increased with pendant side chain length with A6ACA hydrogels showing the maximum calcium content, which then decreased for A8ACA. Moreover, there was an increase in Ca^{2+} content for all hydrogels irrespective of chain length for two immersion cycles as compared to the hydrogels undergoing only 1 immersion cycle (Figure 2.8). (Two-way ANOVA ($\alpha=0.05$); side chain length, $P<0.001$; number of cycles, $P<0.001$; interaction of variables, $P=0.56$). SEM micrographs of the hydrogels revealed the formation of spherical calcium phosphate particles; it was observed that the mineralized phases in A2ACA, A4ABA, A6ACA and A8ACA showed a difference in morphology (Figure 2.9). A2ACA and A4ABA both showed the formation of well defined smooth spherical particles; A6ACA

showed clusters of many highly porous particles of similar diameter and A8ACA showed scattered, irregularly shaped particles on a sheet-like calcium phosphate layer. The Ca/P ratio as determined by EDS analysis, was found to be 1.4, 1.4, 1.6 and 1.6 for mineralized hydrogels synthesized with A2ACA, A4ABA, A6ACA and A8ACA respectively, suggesting again the formation of an apatite-like mineral. XRD analyses revealed that while analysis of phases formed on all hydrogels yielded multiple peaks corresponding to hydroxyapatite (PDF-4-010-6312, based on PDF4+ ICDD database), mineralized phases formed on hydrogels containing A6 and A8 respectively showed greater similarity to hydroxyapatite (Figure 2.10). As in the hydrogels mineralized by immersion in serum/FBS, diffraction peaks those corresponding to halite (NaCl) as well as unidentified peaks, presumably due to ordering within the polymeric hydrogels arising from the PEG chains, were also observed (See Table 2.3 for detailed information regarding the hydroxyapatite peaks identified in the hydrogels). These results support our observation from mineralization studies in serum-supplemented simulated body fluid, wherein longer chain length is more conducive to facilitate the formation of apatite like phases.

2.4. Discussion

The results described here represent a comprehensive and systematic study on mineralization of calcium phosphates on hydrogels with varying pendant side chain lengths (i.e. hydrophobicity), presenting terminal carboxyl functional groups. Binding of Ca^{2+} to the functional groups of the matrix leads to the initial nucleation of mineral phases and is the first step in the templated mineralization of the anionic substrate. It is thus conceivable that any changes that alter the accessibility of terminal carboxyl group

for Ca^{2+} binding could potentially change the matrix assisted templated mineralization process. Studies by Sasaki et al also suggested a potential role of polymer hydrophobicity on co-operative binding of Ca^{2+} to carboxyl residues (Sasaki *et al.*, 1998).

Distinct differences in mineralization were observed depending on the mineralization methods used. The hydrogels that exhibited relatively little mineralization in simulated body fluid showed rapid, extensive mineralization upon immersion in serum-supplemented simulated body fluid (approximately 72 hours in serum-supplemented simulated body fluid, as compared to approximately 3 weeks in serum-free simulated body fluid). This is especially interesting, as the Ca^{2+} and HPO_4^{2-} concentrations were similar in both solutions. Mineralization in serum supplemented simulated body fluid is likely to be mediated through proteins that are adsorbed on hydrogel surfaces. It is important to note that albumin, fibronectin and laminin have been previously reported to play a role in the nucleation of hydroxyapatite (Daculsi *et al.*, 1999; Marques *et al.*, 2002; Oyane *et al.*, 2005; Subburaman *et al.*, 2006). Apparently, certain proteins can have a dual role on the mineralization of apatites, functioning as either a nucleator or inhibitor depending on their conformation and accessibility; these proteins that act as nucleators when adsorbed on a substrate while acting as inhibitors of substrate mineralization when in solution (George and Veis, 2008; He *et al.*, 2003; Oyane *et al.*, 2005; Price *et al.*, 2009).

In order to explain the difference observed in mineralization in the metastable $\text{Ca}^{2+}/\text{HPO}_4^{2-}$ solution in absence of proteins, it is necessary to consider the effect of pendant side chain length on both Ca^{2+} binding and nucleation capacity. As suggested by

Sasaki *et al.*, increases in matrix hydrophobicity tend to promote co-operative Ca^{2+} binding to carboxyl residues (Sasaki *et al.*, 1998). It is also possible that hydroxyapatite formation was favored by longer side chains (or optimal side chain lengths) that support the accessibility of the terminal carboxyl groups at the surface. The increased flexibility in longer side chains could allow the Ca^{2+} bound chains to attain a conformation favoring the formation of apatite crystals through similarity to their nucleated faces. Such conformational mimicking of nucleation faces by ion-binding sites has been implicated as a possible mechanism influencing biomineralization observed in nature (Mann, 1993; Sahai, 2005). The chain length-dependent difference in accessibility could also be a causative factor leading to variation in the morphology of the crystals when the hydrogels were immersed in 40 mM Ca^{2+} /24 mM HPO_4^{2-} . Our findings clearly suggest that A6ACA hydrogels have the optimal side chain length to promote templated mineralization under all the immersion procedures utilized in this study, this is somewhat in agreement with previous studies that demonstrated the ability of A6ACA hydrogels to complex with metal ions and undergo self-organization (Varghese *et al.*, 2001a; Varghese *et al.*, 2001b).

We believe that an important consideration is the accessibility of the carboxyl group to ions in the hydrogel-cation interface, as this accessibility affects Ca^{2+} binding. This is in agreement with previous studies that have shown that the chain length of the pendant side chains of acryloyl amino acid hydrogels plays an important role not only in the extent of binding to transition metal ions, but also plays an important role in modulating the structure of the polymer-metal complex. Significantly, A6ACA was

found to optimally bind to divalent transition metal cations (Varghese *et al.*, 2001a). It is possible that A6ACA hydrogels have an optimal balance between the length of the hydrophobic side chain and the hydrophilic character of the terminal carboxyl group. Solubility calculations involving these hydrogels have suggested that pendant side chains containing more than six groups tend to collapse inward due to increases in side chain hydrophobicity, thereby severely reducing accessibility of the terminal carboxyl groups (Ayala *et al.*, 2011). Thus, the reduced accessibility due to inward collapse of the pendant side chain from A8ACA adequately explains the observed drop in mineralization. It is interesting to note the presence of hydroxyapatite peaks in the XRD spectra for A8ACA hydrogels immersed in serum supplemented SBF despite the lack of observed surface calcification. This could be due to limited nucleation of apatite on the collapsed side chains in the hydrogel interior mediated by the imbibed metal solutions. This conclusion is further supported by the sheetlike morphology of the mineralized phase on A8ACA hydrogels immersed in 40 mM Ca^{2+} /24 mM HPO_4^{2-} suggesting a lack of nucleation sites on the surface.

2.5. Conclusion

We have shown for the first time that small changes in matrix interfacial hydrophobicity (the ability of the matrix to interact with surrounding aqueous milieu) independent of functionality can profoundly influence the Ca^{2+} binding and formation of hydroxyapatite-like mineral phases on hydrogel matrices. While increasing pendant side chain length promotes mineralization upto chains containing five methylene groups, further increase in side chain length can lead to inward collapse of the pendant side chain

and subsequently result in a substantial reduction of apatite nucleation ability. Pendant side chain length can thus affect the extent of mineralization as well as the topology of the mineralized phase. Our findings thus suggest a synergistic effect between functionality and the accessibility of that functionality on the mineralization of polymeric materials, and provide a plausible rationale for the differences in apatite-nucleating ability of osteopontin and bone sialoprotein. The developed approaches can be used to develop mineral-polymer composite materials for use as scaffolds in bone tissue engineering and as bone grafts as well as in other applications requiring the templated synthesis of organic/inorganic composite materials.

2.6. Acknowledgements

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Chapter 2, in full, is a reprint of the material as it appears in “Phadke A *et al.* (2010) Templated mineralization of synthetic hydrogels for bone-like composite materials: role of matrix hydrophobicity. *Biomacromolecules* **11**:2060-2068”. The dissertation author was the primary author on the paper.

2.7. Experimental Section

2.7.1. Synthesis of monomers

N-acryloyl ²⁻glycine (A2AGA), N-acryloyl 4-aminobutyric acid (A4ABA), N-acryloyl 6-aminocaproic acid (A6ACA) and N-acryloyl 8-aminocaprylic acid (A8ACA) were synthesized from glycine (Fisher Scientific Inc), 4-aminobutyric acid, 6-aminocaproic acid and 8-aminocaprylic acid (Acros Organics Inc) respectively, as previously reported (Ayala *et al.*, 2011; Badiger *et al.*, 1998).

2.7.2. Synthesis of PEGDA-6K

In this study, poly (ethylene glycol)-diacrylate (MW: 6000 Da) (PEGDA-6K) was used as a crosslinker and was synthesized from poly (ethylene glycol) (MW: 6000 Da) (Fluka Analytical) as reported previously (Zhang *et al.*, 2009).

2.7.3. Preparation of simulated body fluid (SBF)

Simulated body fluid (m-SBF) was prepared as specified by Oyane *et al.* (Oyane *et al.*, 2003). Briefly, 1L of HEPES-NaOH buffered solution (pH 7.4) in ultrapure water was prepared, containing 142 mM Na⁺, 5 mM K⁺, 1.5 mM Mg²⁺, 2.5 mM Ca²⁺, 103 mM Cl⁻, 10 mM HCO₃⁻, 1.0 mM HPO₄²⁻ and 0.5 mM SO₄²⁻. This was sterilized and filtered using a 0.22 µm sterilizing, low protein binding vacuum filter system (Corning Inc). The solution was stored at 4°C and warmed to 37°C prior to usage.

2.6.4. Preparation of serum-supplemented SBF

SBF was prepared as above. 400 ml of SBF were combined with 100 ml fetal bovine serum (Premium Select, Atlanta Biologicals). This mixture was sterilized and

filtered using a 0.22 μm sterilizing, low protein binding vacuum filter system (Corning Inc). The solution was stored at 4°C and warmed to 37°C prior to usage. All immersions were conducted under sterile conditions.

2.6.5. Preparation of 40 mM Ca^{2+} /24 mM HPO_4^{2-} solution

2.74 g of dipotassium phosphate trihydrate ($\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) (Acros Organics) and 2.22 g CaCl_2 (Acros Organics) were added to 500 ml ultrapure water. 6N HCl was then added dropwise until the disappearance of the resultant turbidity. Prior to usage, 1 M Tris-HCl (pH 7.5) was added dropwise to gradually raise the pH to 5.2, the highest pH at which precipitation was not observed for the duration of the immersion.

2.6.6. Synthesis of hydrogels

0.001 moles of each of the monomers (0.1291 g, 0.157 g, 0.185 g and 0.213 g for A2AGA, A4ABA, A6ACA and A8ACA respectively) was dissolved in 1 ml 1M NaOH to ensure complete deprotonation, thereby obtaining 1 M solution of the sodium salt of each of the monomers. 0.05 g of PEGDA 6K was added as a cross-linker to the solution of each monomer, yielding a final solution containing 5 % PEGDA 6K (w/v). The precursor solutions were subjected to redox polymerization using 0.5% ammonium persulfate as initiator and 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED) as accelerator. This precursor solution was poured into glass moulds and allowed to proceed for 15 minutes at room temperature. The resultant hydrogels measured approximately 0.1 cm in thickness, 8.2 cm in length and 1.5 cm in height. Un-reacted components were leached out of the hydrogels by immersion in DI water for 48 hours with changing the

water intermittently. These hydrogels were then sterilized by immersion in 70% ethanol for 24 hours and air-dried under sterile conditions.

2.6.7. Contact angle measurement

Hydrogels were prepared as mentioned above. Upon synthesis, hydrogels were immersed in DI water for 24 hours to leach out unreacted components and then air-dried at 37°C. In their semi-dried state, hydrogels were confined between two glass slides, and allowed to dry further. This was done in order to prevent curving of the hydrogels during the drying process. The water contact angles of the hydrogels were determined by a sessile drop method at 20°C using goniometer (CAM100, KSV Instruments Ltd.). A 5µL droplet of water was placed on the surface of hydrogels, following which the droplets were imaged and the angles were calculated using CAM100 software. All samples were prepared as triplicates and results were shown as a mean value with standard deviation.

2.6.8. Mineralization of hydrogels by in protein-supplemented SBF

Dried hydrogels were equilibrium swollen in SBF. The hydrogels (n=3 for each monomer) were immersed in serum-supplemented simulated body fluid for a period of two weeks, with daily exchange of solution to ensure continuous supply of ions.

2.6.9. Mineralization of hydrogels in 40 mM Ca^{2+} /24 mM HPO_4^{2-} solution

Dried hydrogels (n=3 for each monomer) were equilibrium swollen in SBF and were then immersed in 40 mM Ca^{2+} /24 mM HPO_4^{2-} for 30 minutes and then rinsed with DI water. These were then immersed in simulated body fluid for 24 hours- this entire process was considered as one immersion cycle. Hydrogels were mineralized through one and two such immersion cycles respectively.

2.6.10. Adsorption of serum proteins on hydrogel surfaces

The hydrogels (n=3 for each monomer) were immersed in fetal bovine serum (Premium Select, Atlanta Biologicals) for 4 hours at 37°C. They were then transferred to a fresh vessel and washed with Laemmli sample buffer (Laemmli, 1970)- this was to ensure that proteins eluted on the sample buffer were proteins adsorbed on the gels and not the surface on which immersion with FBS was carried out. Solutions collected from replicates for each monomer were pooled and subjected to electrophoresis with SDS-PAGE. Proteins were visualized through staining with Coomassie Brilliant Blue G-250 of the polyacrylamide gels following electrophoresis.

2.6.11. SEM-EDS

Following mineralization, the hydrogels were thoroughly rinsed with running DI water. They were then flash-frozen with liquid N₂ and lyophilized for characterization. Lyophilized hydrogels were sputter coated with Cr (Denton Desk IV Sputter Coater) and imaged using Phillips XL30 ESEM to study microstructural topology and composition of hydrogel-mineral composites was studied using Oxford EDX attachment and INCA analysis software.

2.6.12. X-Ray Diffraction

Following mineralization, the hydrogels were thoroughly rinsed with running DI water. They were then flash-frozen with liquid N₂ and lyophilized for characterization. Lyophilized mineral-hydrogel composites were powdered and analyzed by powder X-ray diffraction using a Rigaku RU200Bh DMax-RB rotating anode diffractometer. The X-ray source was a Cu anode generating Cu K_{α1} x-rays ($\lambda=0.154056$ nm). The diffractometer

was operated at 40 kV beam energy and 120 mA beam current. A graphite monochromator [0002] orientation ($2d=0.6708$ nm) was used and the diffracted beam was collected into a horizontal goniometer covering an angular range (2θ) from 5° to 80° . The collected spectra were analyzed using MDI Jade X-Ray analysis software employing PDF-4+ ICDD database for search match peak identification.

2.6.13. Measurement of Ca^{2+} content in mineralized phase

Following mineralization, the hydrogels were thoroughly rinsed with running DI water. They were then flash-frozen with liquid N_2 and lyophilized for characterization. For gels mineralized through immersion in serum-supplemented SBF, each lyophilized gel ($n=3$ for each monomer) was homogenized in 0.5 ml 0.5 N HCl; for gels mineralized through immersion in 40 mM Ca^{2+} /24 mM HPO_4^{2-} , each lyophilized gel was homogenized in 1 ml 0.5 N HCL.

This homogenate was vigorously vortexed for 24 hours at 4°C in order to completely dissolve the calcium phosphate mineral. The Ca^{2+} concentration in this solution was measured by spectrophotometric analysis with cresolphthalein complexone, using Calcium Reagent (two part liquid) set (Pointe Scientific Inc) and used to determine the Ca^{2+} content in the original lyophilized sample as described elsewhere (Varghese *et al.*, 2010). The calculated Ca^{2+} content was normalized to the dry weight of the sample.

2.6.14. Statistical analysis

For immersion in serum-supplemented simulated body fluid, statistical analysis was carried out using one way ANOVA; for immersion in 40 mM Ca^{2+} /24 mM HPO_4^{2-} , two-way ANOVA was used. Microsoft Excel 2007 was used for statistical analysis.

2.7. Figures

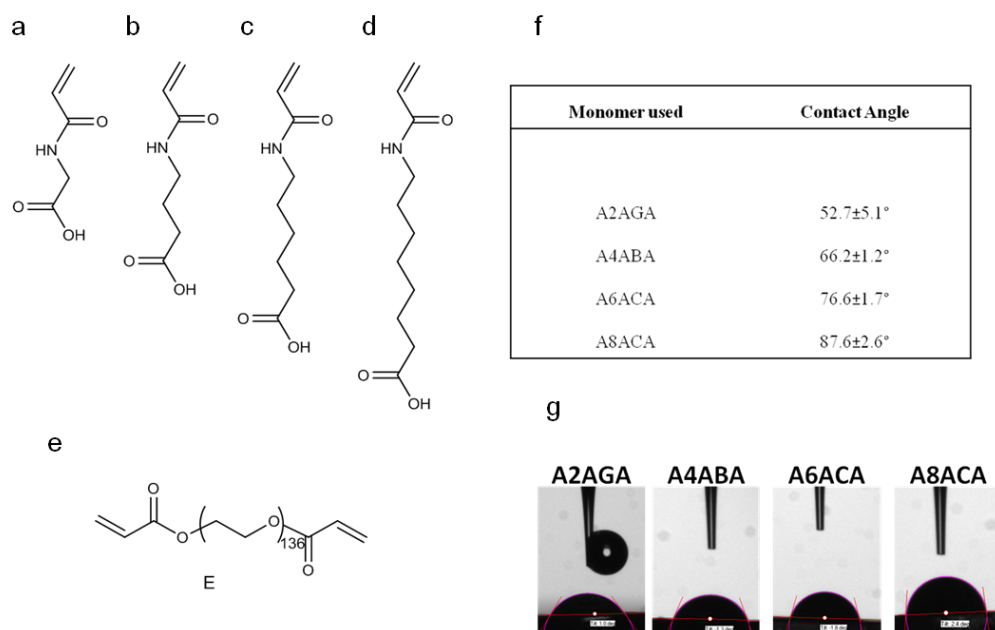


Figure 2.1. Synthesis of hydrogels with varying pendant side chain length

(a-f) Structures of N-acryloyl amino acids used in the study, namely (a) A2ACA (b) A4ABA (c) A6ACA (d) A8ACA and (e) cross-linker, PEGDA 6K. (f) Contact angles of the hydrogels of varying side chain lengths and (g) images of the water droplets on the various hydrogels during contact angle measurement.

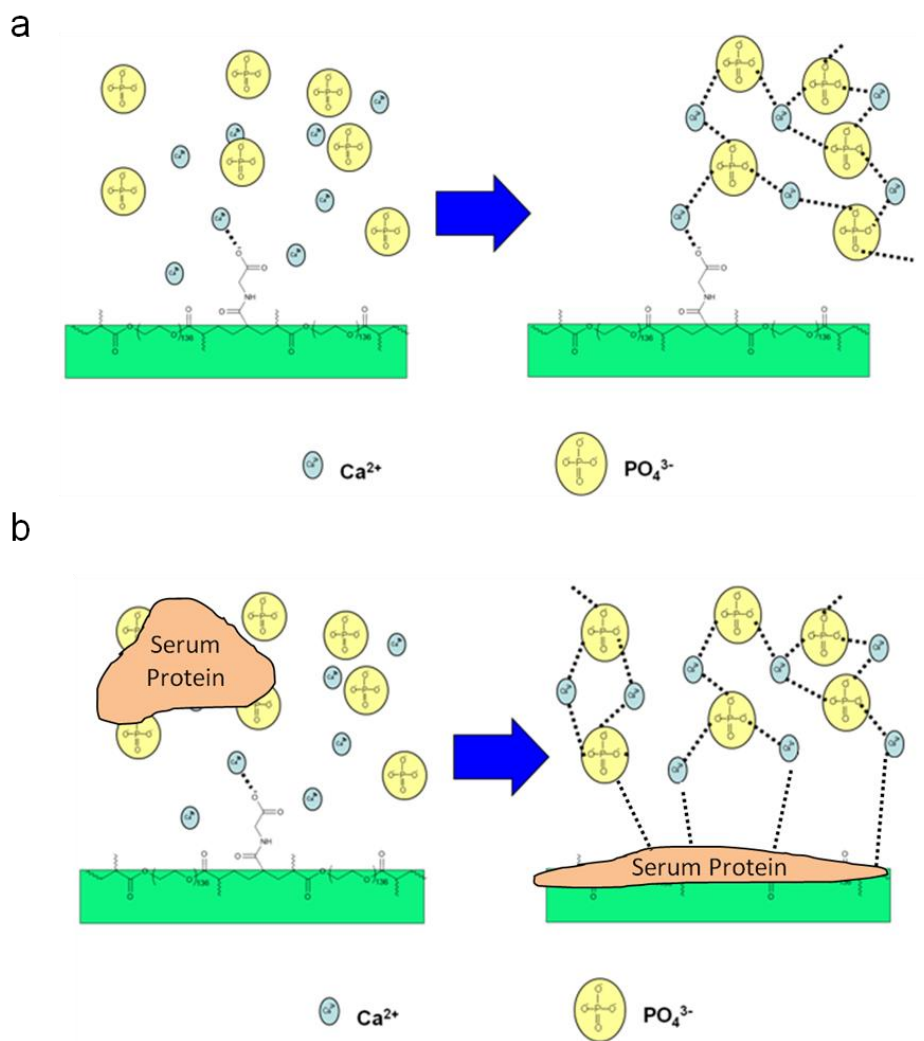


Figure 2.2. Schematic depiction of mineralization of hydrogels

Mineralization of hydrogels under (a) protein-free conditions and (b) in the presence of serum proteins

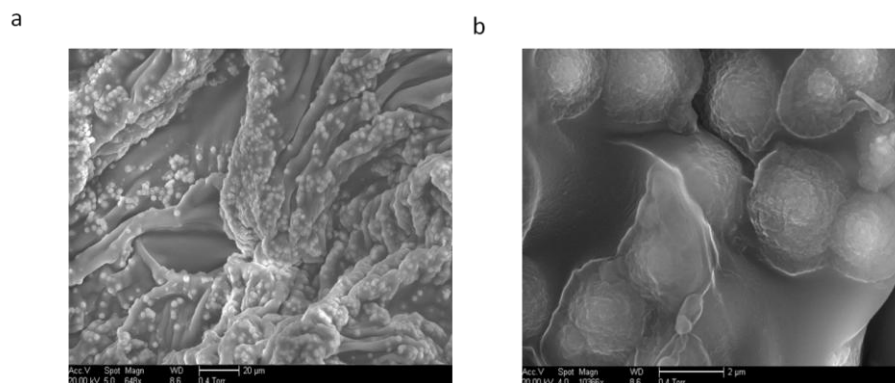


Figure 2.3. A6ACA hydrogels mineralized through immersion in m-SBF

SEM images of A6ACA hydrogels immersed for 3 weeks in SBF at (a) low magnification and (b) high magnification, showing the formation of calcium phosphate spherulites

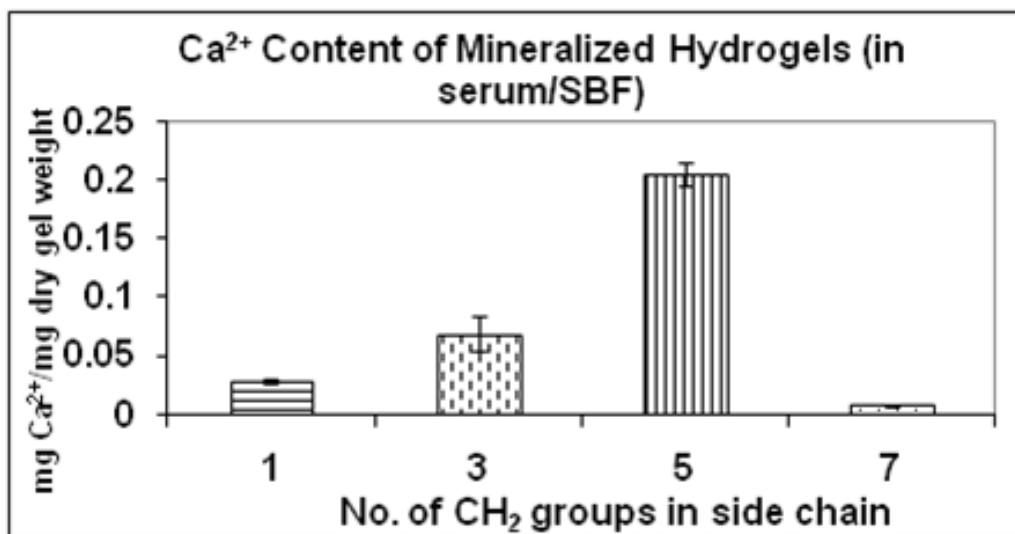


Figure 2.4. Ca²⁺ content of hydrogels mineralized in serum-SBF

Ca²⁺ concentration in hydrogels of varying side chain lengths, mineralized by immersion in SBF/10% FBS. Error bars represent standard deviation (n=3)

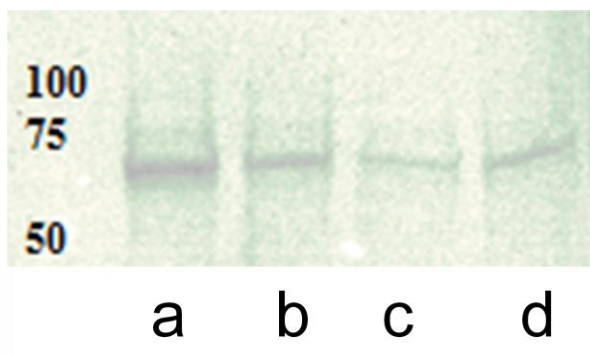


Figure 2.5. Protein adsorption on hydrogels

Coomassie blue staining showing adsorption of serum proteins on (a) A2AGA (b) A4ABA (c) A6ACA and (d) A8ACA

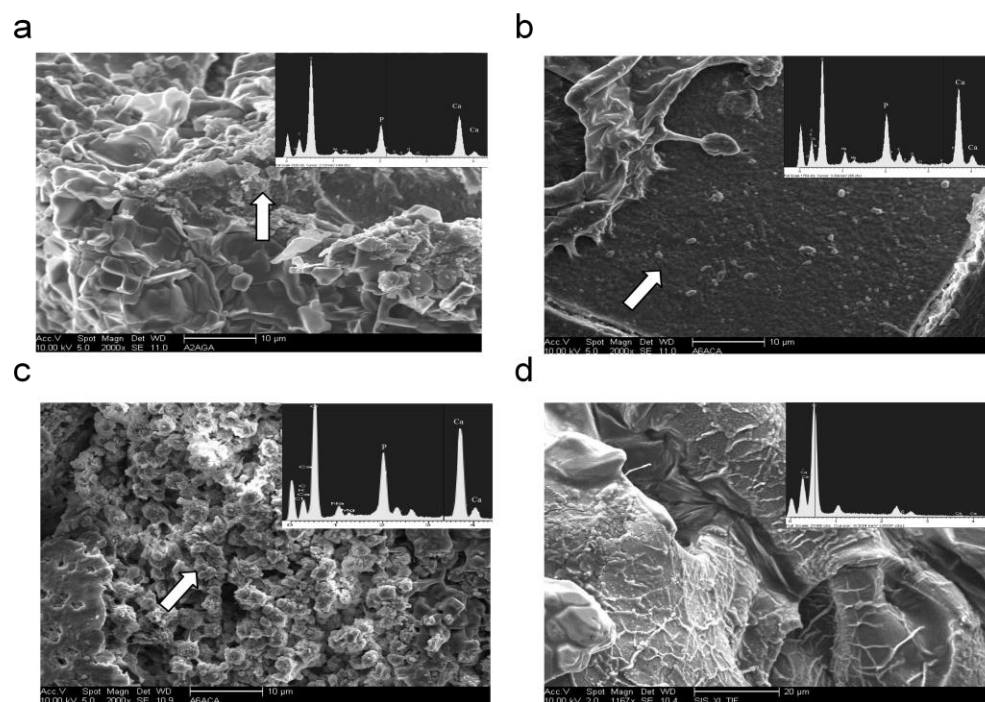


Figure 2.6. Morphology of hydrogels mineralized in presence of proteins

SEM micrographs with energy dispersion spectra (inset) of hydrogels containing (a) A2AGA, (b) A4ABA, (c) A6ACA and (d) A8ACA mineralized by immersion in serum supplemented simulated body fluid. Note that (d) shows the absence of any observable deposits containing calcium phosphate as seen in EDS. Arrows indicate calcium phosphate particles.

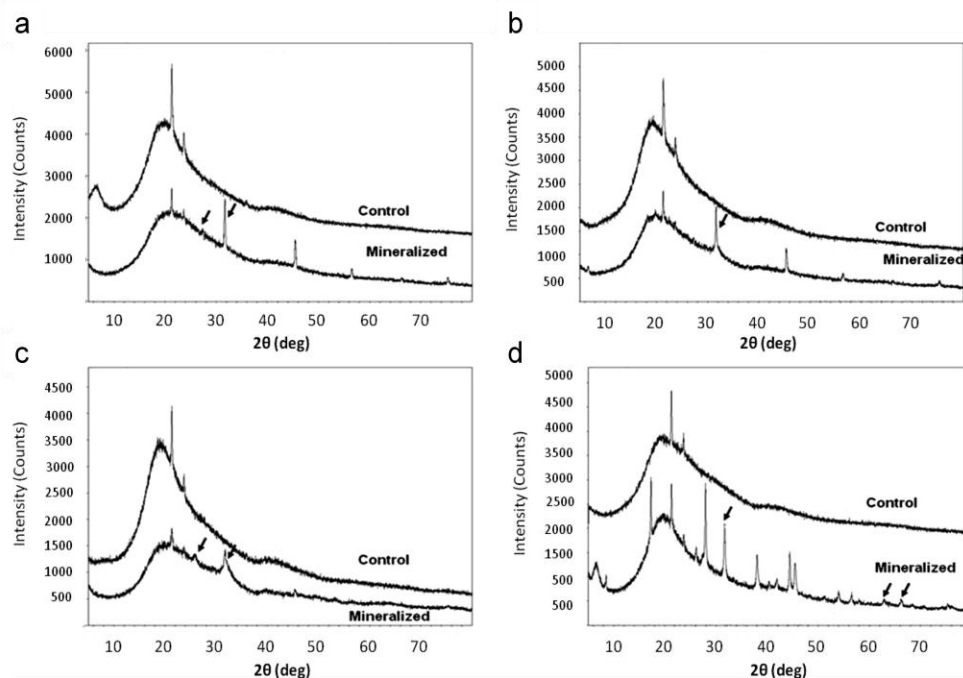


Figure 2.7. XRD spectra of hydrogels mineralized in serum-SBF

X-ray diffraction spectra of hydrogels mineralized by immersion in serum-supplemented simulated body fluid, containing (a) A2AGA (b) A4ABA (c) A6ACA (d) A8ACA, with a control, non-mineralized hydrogel for each sample for comparison. Arrows indicate peaks corresponding to hydroxyapatite (PDF-04-010-6312 in PDF-4+ database)

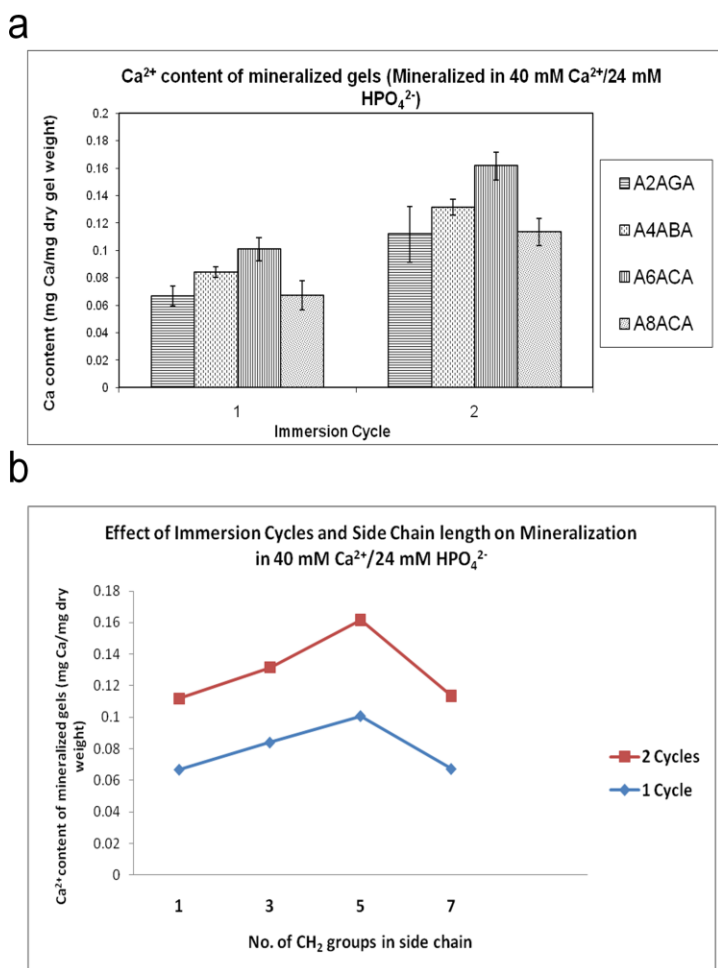


Figure 2.8. Mineralization of hydrogels in 40 mM Ca²⁺/24 mM HPO₄²⁻

Ca²⁺ content of hydrogels mineralized by immersion in 40 mM Ca²⁺/24 mM HPO₄²⁻ after 1 and 2 cycles respectively. (a) Ca²⁺ content of the hydrogels as a function of pendant side chain length and number of immersion cycles. Error bars represent standard deviation (n=3). (b) Graph demonstrating that the effect of pendant side chain length on Ca²⁺ content is maintained across multiple immersion cycles.

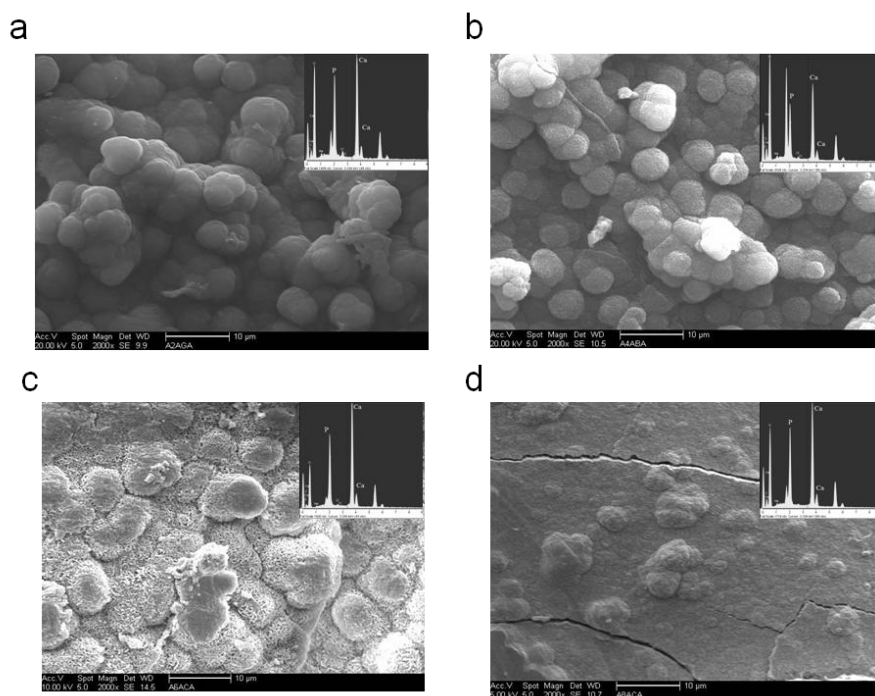


Figure 2.9. Morphology of hydrogels mineralized in 40 mM Ca^{2+} /24 mM HPO_4^{2-}

Scanning electron microscopy images and energy dispersion spectra (inset) of (a) A2AGA (b) A4ABA (c) A6ACA and (d) A8ACA-containing hydrogels mineralized for 2 cycles, by immersion in 40 mM Ca^{2+} /24 mM HPO_4^{2-}

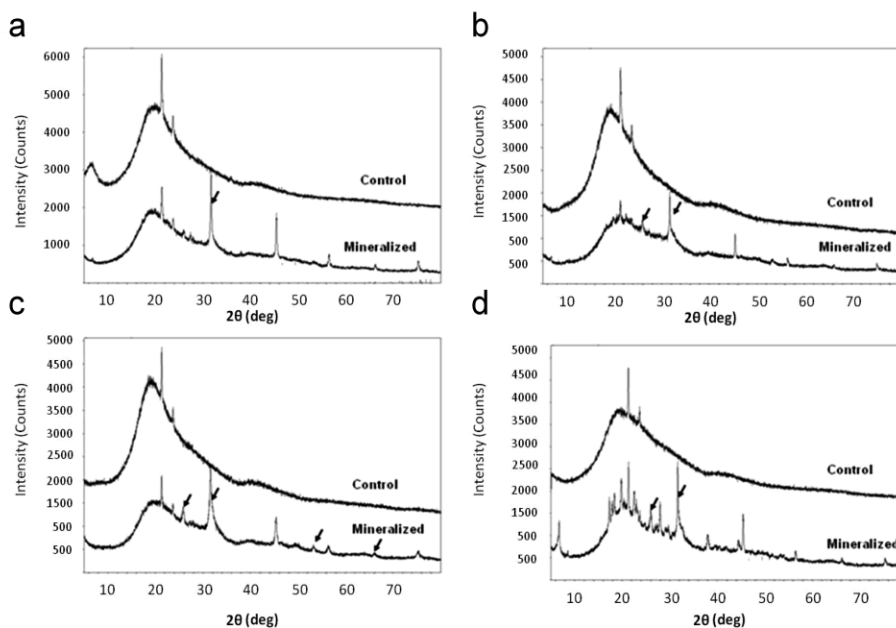


Figure 2.10. XRD spectra for hydrogels mineralized in 40 mM Ca²⁺/24 mM HPO₄²⁻

X-ray diffraction spectra for hydrogels mineralized in 40 mM Ca²⁺/24 mM HPO₄²⁻, containing (a) A2AGA (b) A4ABA (c) A6ACA (d) A8ACA with a control, non-mineralized hydrogel for comparison. Arrows represent peaks corresponding to hydroxyapatite (PDF-04-010-6312 in PDF-4+ database)

2.8. Tables

Table 2.1. Apatite peaks in hydrogels mineralized with serum- SBF

Monomer used	Number of Methylene Groups in Side Chain	Hydroxyapatite peaks identified		Relative Intensity	
		2 θ	d(Å) Phase	Peak Height / Background	%H (Relative to most intense peak)
A2AGA	1	25.943	3.4420	540	52.2
		31.739	2.8265	1162	100
A4ABA	3	31.659	2.8265	945	100
A6ACA	5	25.778	3.4420	150	39.2
		31.682	2.8265	311	81.2
A8ACA	7	38.040	2.3676	711	42.3
		62.840	1.4807	122	7.2
		66.160	1.4133	154	9.2

Table 2.2. Monomers used to vary matrix hydrophobicity

Monomer	No. of Methylene groups in side chain	Parent Amino Acid	pK₁ (Ref) of terminal carboxyl
A2AGA	1	Glycine	2.34 (Svedas <i>et al.</i> , 1980)
A4ABA	3	4-Aminobutyric Acid	4.23 (Dawson <i>et al.</i> , 1989)
A6ACA	5	6-Aminocaproic Acid	4.37 (Edsall <i>et al.</i> , 1958)
A8ACA	7	8-Aminocaprylic Acid	4.62 (Kitano <i>et al.</i> , 2005)

Table 2.3. Apatite peaks in hydrogels mineralized in 40 mM Ca^{2+} /24 mM HPO_4^{2-}

Monomer Used	Number of Methylene Groups in Side Chain	Hydroxyapatite peaks identified		Relative Intensity	
		2 θ	d(Å) Phase	Peak Height / Background	%H (Relative to most intense peak)
A2AGA	1	31.739	2.8265	1889	100
A4ABA	3	25.999	3.442	235	23.7
		31.719	2.8265	900	100
A6ACA	5	25.916	3.4420	344	28.4
		31.620	2.8265	1210	100
		53.182	1.7210	128	10.6
		66.059	1.4133	72	5.9
A8ACA	7	22.520	3.9013	560	35.1
		38.000	2.3676	341	21.4

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CHAPTER 3: Mineralized synthetic matrices as an instructive microenvironment for osteogenic differentiation of human mesenchymal stem cells

3.1. Abstract

Mesenchymal stem cells (MSCs) are multipotent progenitors and can differentiate into bone, cartilage, fat, and muscle; for controlling the differentiation of these cells, it is important to design instructive microenvironments with required physicochemical cues. Of these, the extracellular matrix-based cues (insoluble component of the microenvironment) play an important role in directing tissue specific differentiation of stem cells. In this study, the effect of substrate-mediated signals on osteogenic differentiation of human MSCs (hMSCs) was investigated by using a synthetic bone-like composite material comprising of both organic and inorganic components. This synthetic material, developed through templated mineralization, supported adhesion, spreading, and proliferation of hMSCs. Additionally, hMSCs on these materials underwent osteogenic differentiation even in the absence of osteogenesis-inducing supplements such as β -glycerolphosphate and dexamethasone. HMSCs on the mineralized hydrogels exhibited higher expressions of Runx2, bone sialoprotein, and osteocalcin compared to their matrix-rigidity matched, non-mineralized hydrogel counterparts, and cell culture coverslips. The mineralized hydrogel-assisted osteogenic differentiation of hMSCs could be attributed to their exposure to high local concentrations of calcium and phosphate ions (due to dissolution of the mineral layer) in conjunction with chemical and topographical

cues arising from the hydrogel-bound calcium phosphate mineral layer. Beyond demonstrating osteogenic differentiation of hMSCs by employing synthetic materials having a bone-like composite structure, this study also shows how matrices can provide both insoluble and soluble cues to promote osteogenesis of hMSCs.

3.2. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into osteogenic, chondrogenic, adipogenic, and myogenic lineages and have been derived from a variety of adult tissues (Caplan, 1991; Hwang *et al.*, 2009; Jaiswal *et al.*, 1997; Pittenger *et al.*, 1999). Traditionally, *in vitro* osteogenesis of MSCs is achieved by culture with osteogenesis-inducing medium containing dexamethasone, ascorbic acid, and β -glycerolphosphate (Jaiswal *et al.*, 1997). Recent research has demonstrated the role of physicochemical properties of the bone-specific extracellular matrix in mediating osteogenic differentiation. A number of material properties such as surface topography (Dalby *et al.*, 2007) matrix stiffness (Engler *et al.*, 2006) hydrophobicity (Ayala *et al.*, 2011) and surface chemistry (Benoit *et al.*, 2008) have been shown to play an important role in directing osteogenic differentiation of stem cells. Bone consists of an inorganic apatite-like mineral phase as well as an organic protein phase (known as osteoid) composed primarily of type I collagen, proteoglycans, and non-collagenous proteins (Weiner and Wagner, 1998). While the exact mechanism responsible for the mineralization of the osteoid is unclear, it is believed that regions rich in charged residues such as aspartate and glutamate bind to Ca^{2+} ions, leading to nucleation of the apatite-like phase (Boskey, 1996; Boskey, 1995; George and Veis, 2008; Ito *et al.*, 2004; Palmer *et*

al., 2008) This composite structure of bone, consisting of organic and inorganic components, plays an important role in its homeostasis and function (Palmer *et al.*, 2008).

Several naturally derived and synthetic materials have been shown to support osteogenesis of hMSCs through providing both, structural support as well as specific physicochemical cues (Chastain *et al.*, 2006; Farrell *et al.*, 2006; Mauney *et al.*, 2004; Yang *et al.*, 2001). Another approach for stimulating osteogenic differentiation utilizes inorganic minerals, emulating the mineralized components of bone. In particular, calcium phosphate-based minerals have been used as osteoinductive substrates (Mygind *et al.*, 2007; Yuan *et al.*, 1998) *In vitro*, these materials have been shown to promote osteogenic differentiation of progenitor cells (Kotobuki *et al.*, 2005; LeGeros, 2008; Müller *et al.*, 2008; Mygind *et al.*, 2007; Toquet *et al.*, 1999) Bioactive ceramics such as bioglasses and calcium phosphates have been shown to form a surface layer of apatite-like mineral in simulated body fluid (Ducheyne and Qiu, 1999). When combined with stem cells, these materials have been shown to promote healing of critically sized bone defects (Kadiyala *et al.*, 1997) both through promoting infiltration by host osteoblastic and progenitor cells followed by their osteogenic differentiation (Moore *et al.*, 2001). A shortcoming of such systems however, is poor adhesion between the newly formed apatite layer and the native ceramic, leading to eventual delamination between the two layers (Saiz *et al.*, 2002).

In an effort to develop synthetic matrices integrating both organic and inorganic components of native bone tissue, studies have incorporated inorganic minerals into organic materials. Indeed, such organic/inorganic composites were found to promote osteogenic differentiation of progenitor cells (Kim *et al.*, 2006; Takahashi *et al.*, 2005)

and have also been shown to promote healing of critically sized bone defects (Cowan *et al.*, 2004; Gauthier *et al.*, 2001; Gauthier *et al.*, 2005). Such systems devoid of any molecular level mixing or binding between the two components could suffer from the lack of a strong interface between the organic and inorganic phases. Studies have shown that templated mineralization mimicking various attributes of biomineralization could improve the organic-mineral interfacial strength (Ball *et al.*, 2005; Gkioni *et al.*, 2010; Kretlow and Mikos, 2007; Ngankam *et al.*, 2000; Palmer *et al.*, 2008; Rezwan *et al.*, 2006; Song *et al.*, 2005; Song *et al.*, 2003; Suzuki *et al.*, 2006). Templated mineralization typically utilizes charged groups of the organic materials such as carboxyl, hydroxyl, and imine groups that are capable of binding to Ca^{2+} or PO_4^{3-} ions, thereby initiating nucleation of calcium phosphates (Kretlow and Mikos, 2007; Shkilnyy *et al.*, 2008; Song *et al.*, 2005; Song *et al.*, 2003). In fact studies also suggest that templated mineralization of materials provides superior osteoinductivity to merely introducing hydroxyapatite as additives within the material (Osathanon *et al.*, 2008). Such materials that can simultaneously present several physicochemical cues and act as a reservoir of inorganic Ca^{2+} and PO_4^{3-} ions could be optimal substrates to direct osteogenic differentiation of progenitor cells.

In a previous study, we have developed hydrogel/mineral composites through templated mineralization of polyanionic hydrogels (Phadke *et al.*, 2010b), where poly(ethylene glycol) (PEG) hydrogels were modified with dangling side chain moieties of varying length terminating in a carboxyl group. In particular, hydrogels containing N-acryloyl 6-aminocaproic acid (A6ACA) moieties (PEG/A6ACA) were found to undergo

optimal mineralization. In the current study, we investigated the potential of mineralized PEG/A6ACA hydrogels as instructive materials for promoting osteogenic differentiation of hMSCs.

3.3. Results and Discussion

3.3.1. Material characterization

PEG/A6ACA hydrogels were mineralized through immersion in calcium phosphate solution; Ca^{2+} binding to the terminal carboxyl groups of A6ACA promoted the subsequent nucleation of a calcium phosphate phase. Mineralized hydrogels appeared white in color, in comparison to non-mineralized hydrogels, which were transparent in appearance (Figure 3.1a,b). Measurement of calcium concentration confirmed the calcification of mineralized hydrogels, which contained 98 ± 2 mg Ca^{2+} /g dry weight, as compared to non-mineralized hydrogels which contained 2 ± 1 mg Ca^{2+} /g dry weight (Figure 3.1c). Scanning electron microscopy of mineralized hydrogels showed irregularly shaped spherulites roughly $0.5 \mu\text{m}$ in diameter (Figure 3.1d), not observed in non-mineralized hydrogels (Figure 3.1e). Elemental analysis showed that these minerals consisted chiefly of calcium phosphate with a Ca/P ratio of approximately 1.5; this is close to the Ca/P ratio observed in other bioactive ceramics such as β -tricalcium phosphate (1.5) and hydroxyapatite (1.67) (Raynaud *et al.*, 2002).

The mineralized hydrogels were found to have a compressive modulus of 31 ± 4 kPa, compared to 17 ± 2 kPa for their non-mineralized counterparts, suggesting that the mineralization of the hydrogels did indeed increase their stiffness as anticipated. To account for this effect, non-mineralized hydrogels with increased stiffness were created

by tuning the concentration of PEGDA6K so as to match the compressive modulus of the mineralized hydrogels. Non-mineralized hydrogels containing 4% PEGDA6K had a compressive modulus of 31 ± 3 kPa, thus showing a compressive modulus very similar to that of mineralized hydrogels. This matrix-rigidity matched, non-mineralized hydrogels were used subsequently as controls to account for the effect of rigidity due to mineralization. Here after these hydrogels will be merely referred to as non-mineralized hydrogels.

3.3.2. Cell attachment

Chemical and physical properties of the matrix play an important role in mediating initial adhesion of cells to the underlying substrate, which in turn have an important role in stem cell differentiation (Phadke *et al.*, 2010a). Adhesion of hMSCs to mineralized hydrogels were examined and compared against that on rigidity matched non-mineralized hydrogels and cell culture coverslips. These coverslips, which are known to promote cell attachment, were used because of their usage in cell culture as negative control substrates (Ayala *et al.*, 2011; Hu *et al.*, 2007).

Cells were found to attach to all the substrates. Initial attachment was observed within 12 hours of seeding on both the hydrogels, although a large number of cells displayed a round morphology. After 2 days of culture, analysis of cell morphology yielded a shape index of 0.2 ± 0.1 on the mineralized hydrogels and 0.2 ± 0.1 on the non-mineralized hydrogels, suggesting a somewhat elongated morphology on both, mineralized and non-mineralized hydrogels. HMSCs on coverslips yielded a similar shape index (0.2 ± 0.1). A shape index of 1 indicates round morphology with lower shape

index values suggestive of elongated morphology. Intriguingly, both mineralized and non-mineralized hydrogels as well as coverslip controls showed very similar DNA content after 7 and 14 days of culture (Figure 3.2a). The nearly two-fold increase in DNA content for both mineralized and non-mineralized hydrogels as well as coverslips suggests that all surfaces supported similar cell proliferation.

The hMSCs on both the mineralized and non-mineralized hydrogels exhibited similar proliferation albeit with differences. Cells on the non-mineralized hydrogels and coverslips uniformly populated the surface of the hydrogels (Figure 3.2b, d) at 2 days and were 100% confluent at 14 days (Figure 3.2e, g). Though the cells on mineralized hydrogels initially showed uniform attachment and spreading on the surface at 2 days (Figure 3.2c), they showed significantly different behavior. These cells formed highly condensed regions soon after reaching confluence, leading to highly non-homogenous distribution of cells over the sample surface at 14 days (Figure 3.2f). Such aggregation has previously been noted in the osteogenic differentiation of hMSCs using traditional osteogenic stimuli in culture medium (Jaiswal *et al.*, 1997).

F-actin staining revealed differences in the alignment of stress fibers on mineralized and non-mineralized hydrogels and coverslips. Cells on coverslips and non-mineralized controls showed aligned, large stress fibers between adjacent cells that spanned the entire cell (Figure 3.3a, c, d, f). On mineralized hydrogels, the cytoskeleton appeared to contain thinner, shorter stress fibers as compared to non-mineralized hydrogels and coverslip controls; additionally the stress fibers appeared to be more prominent at the cell periphery (Figure 3.3b, e). Such cytoskeletal re-ordering of stress

fibers has been previously reported during the osteogenic differentiation of hMSCs using traditional medium stimuli (Titushkin and Cho, 2007) suggesting that the mineralized hydrogels could promote osteogenesis of hMSCs.

3.3.3. Osteogenic differentiation

Having established that the mineralized matrices support adhesion, growth, and cytoskeletal re-organization in hMSCs, we next determined the effect of mineralized matrices on their osteogenic differentiation in order to evaluate their osteoinductive capacity. Quantitative RT-PCR (qPCR) revealed upregulation of osteogenic genes, namely, Runx2 (RUNX2), osteocalcin (OCN) and bone sialoprotein (BSP) in mineralized hydrogels, as compared to non-mineralized hydrogels as well as coverslip controls (Figure 3.4). OCN showed increased upregulation on the mineralized hydrogels compared to coverslips and non-mineralized hydrogels at both, 7 and 14 days. BSP on the other hand, showed significant upregulation in the mineralized hydrogels at 14 days. OCN and BSP are specific to the osteoblastic phenotype and are associated with the mineralized matrix; their increased expression indicates osteogenic differentiation of hMSCs on the mineralized hydrogels. Runx2 on the other hand, is a transcription factor that serves as an early osteogenic marker. It is possible that the presence of a mineralized environment stimulated the production of mineralization-specific proteins, leading to upregulation of OCN and BSP on mineralized hydrogels.

Evidence for osteogenic differentiation at the protein expression level was obtained through quantitative measurement of alkaline phosphatase (ALP) activity, as ALP is an early marker for osteogenesis. Cells on mineralized hydrogels showed the

highest ALP activity, while cells on coverslips and non-mineralized hydrogels showed similar, lower activity further providing evidence of osteogenic differentiation (Figure 3.5a). It is important to note that as the entire sample was homogenized in the sample collection buffer, the samples collected from mineralized hydrogels could contain increased levels of inorganic phosphate which is a well-known competitive inhibitor of ALP activity (Fernley and Walker, 1967). It is thus likely that the actual ALP activity on the mineralized samples is even higher than the measured activity.

Osteogenic differentiation of hMSCs on various substrates was further assessed by immunofluorescent staining for OCN. For cells seeded on coverslips, no visible staining was observed at 14 days (Figure 3.5b); hMSCs on mineralized hydrogels stained positive for OCN (Figure 3.5c) while cells on non-mineralized hydrogels showed extremely weak staining (Figure 3.5d). Samples stained at 21 days showed bright OCN staining on the mineralized hydrogels (Figure 3.5f), with extremely weak OCN staining on coverslips and non-mineralized hydrogels (Figures 5e,g).

Another matrix-associated parameter that has been shown to influence osteogenic differentiation is the topography. As observed in Figure 1d and 1e, mineralized hydrogels present a vastly different surface topography as compared to non-mineralized hydrogels; the rough topography presented by the mineralized hydrogels could also contribute to the osteogenic differentiation of hMSCs (Dalby *et al.*, 2007).

In addition to the matrix-based insoluble cues, another factor contributing to the mineralized hydrogel-mediated osteogenic differentiation of hMSCs could be the local concentration of Ca^{2+} and PO_4^{3-} due to dissolution of the calcium phosphate layer of the

mineralized hydrogels. Calcium phosphates of the mineralized layer can release soluble Ca^{2+} and PO_4^{3-} while undergoing dissolution, thus increasing their local concentrations. Higher levels of Ca^{2+} and PO_4^{3-} have been demonstrated to promote osteogenesis of progenitor cells (Chai *et al.*, 2010; Rashid *et al.*, 2003). This dissolution of the mineralized phase is a dynamic process where the released Ca^{2+} and PO_4^{3-} could lead to spontaneous precipitation of calcium phosphate due to local increase in Ca^{2+} and PO_4^{3-} concentrations (Lu and Leng, 2005; Radin and Ducheyne, 1993). Studies have shown that the precipitation of calcium phosphate can lead to co-precipitation of osteoinductive factors such as bone morphogenic proteins, which also have high affinity for apatite-like calcium phosphates (Liu *et al.*, 2004; ter Brugge *et al.*, 2002; Urist *et al.*, 1984). Such behavior has been previously demonstrated in bioactive ceramics (Ducheyne *et al.*, 1993; Radin and Ducheyne, 1993; ter Brugge *et al.*, 2002; Yao *et al.*, 2010). Higher levels of Ca^{2+} and PO_4^{3-} in serum-based medium have been shown to form fetuin-calcium phosphate complexes (Price and Lim, 2003). These complexes have been shown to play an important role in bone tissue maintenance (Jahnen-Dechent *et al.*, 2011; Price *et al.*, 2002) and could also influence osteogenic differentiation of progenitor cells. To this end, we determined the release of Ca^{2+} and PO_4^{3-} from acellular mineralized hydrogels in Ca^{2+} - and PO_4^{3-} -free 50 mM Tris buffer at physiological pH of 7.4. As anticipated, the mineralized hydrogels released both Ca^{2+} and PO_4^{3-} , where the Ca^{2+} and PO_4^{3-} showed similar release profile (Figure 3.6a, b). Moreover, the molar ratio between the released Ca^{2+} and PO_4^{3-} was 1.7 ± 0.2 (averaged over 7 days). This is very close to the stoichiometric Ca/P ratio in hydroxyapatite (1.67) suggesting that the Ca^{2+} and PO_4^{3-}

release could be due to the dissolution of an apatite-like calcium phosphate phase from the mineralized hydrogels.

To determine whether increase in soluble Ca^{2+} and PO_4^{3-} concentrations could promote osteogenic differentiation, we exposed hMSCs seeded on coverslip controls to growth medium supplemented with increased levels of Ca^{2+} and PO_4^{3-} respectively; indeed, we observed upregulation of osterix (OSX) and Runx2 and to a smaller extent, OCN, as compared to hMSCs cultured in control growth medium for up to 7 days (Figure 3.6c-e). The gene expression data further indicates that Ca^{2+} -supplemented medium promoted the expression of OSX and Runx2 to a greater extent than PO_4^{3-} -supplemented medium. Taken together, these data suggest that increased levels Ca^{2+} and PO_4^{3-} ions arising from dissolution of the calcium phosphate on the mineralized hydrogels could play a role in promoting osteogenic differentiation of hMSCs. Our results are in agreement with a recent study by Chai *et al.* (Chai *et al.*, 2010) which also probed dynamic dissolution of calcium phosphates as a potential mechanism behind the osteoinductivity of calcium phosphate-based materials. Interestingly, supplementation of the culture medium with Ca^{2+} and PO_4^{3-} was mainly able to stimulate expression of early osteogenic markers (viz. Runx2 and OSX) although higher Ca^{2+} concentration did result in increased OCN expression at 3 days while higher PO_4^{3-} concentration promoted increased OCN at 7 days. This is in contrast with the mineralized hydrogels, which were able to induce significantly higher upregulation of OCN expression at both 7 and 14 days.

Together the results discussed above suggest that mineralized PEG/A6ACA hydrogels can direct osteogenic differentiation of hMSCs, thus promising osteoinductive

substrates, via a combination of physicochemical cues (*e.g.* topology and insoluble chemical cues from the calcium phosphate) and soluble cues (*e.g.* increased local Ca^{2+} and PO_4^{3-} concentrations as a result of dissolution of calcium phosphates).

3.4. Conclusion

We have demonstrated the ability of bone-mimetic PEG/A6ACA hydrogels mineralized through a templated process to promote the attachment, spreading and proliferation of hMSCs. These materials were found to promote osteogenic differentiation of hMSCs even in the absence of osteogenesis-inducing medium supplements such as dexamethasone and β -glycerolphosphate, suggesting the potential of these materials to provide an instructive environment conducive for osteogenic differentiation of stem cells. We also probed the contribution of potential parameters to this osteoinductivity and found that a combination of factors such as topology, chemical cues from the material-bound minerals and dynamic mineral dissolution were likely responsible. This study thus demonstrates the development of an osteoinductive synthetic material containing bone-like calcium phosphate mineral as a promising candidate for bone tissue repair.

3.5. Experimental Section

3.5.1. Hydrogel synthesis

N-acryloyl 6-aminocaproic acid (A6ACA) and poly(ethylene glycol) (Mn: 6 kDa)-diacrylate (PEGDA 6K) were synthesized as detailed previously (Phadke *et al.*, 2010b). A6ACA was dissolved in 1N sodium hydroxide to obtain a 1M solution (185 mg/ml) to which PEGDA6K was added to obtain a 2% solution of PEGDA6K (20

mg/ml). This precursor solution was polymerized using 0.5% ammonium persulfate as initiator and 0.15% N,N,N',N' tetramethylethylenediamine (TEMED) as accelerator in Bio-Rad 1 mm spacer glass molds for 15 minutes at 25°C. To create matrix rigidity matched non-mineralized hydrogels, the above procedure was repeated, except using 4% PEGDA 6K in the precursor solution instead of 2% as detailed above. The N-acryloyl 6-aminocaproic acid-co-poly(ethylene glycol) (PEG/A6ACA) hydrogels were then incubated in phosphate buffered saline (PBS; pH 7.4) at 25°C for 18 hours, with two changes of buffer. The hydrogel sheets were then cut into discs measuring 1 cm² in area. The cut hydrogel disks were further immersed in PBS for 24 hours at 37°C with 3X solution change to remove un-reacted reagents.

3.5.2. Preparation of Hydrogel-Mineral composites

Hydrogel discs were mineralized by a method similar to a previously described procedure (Phadke *et al.*, 2010b) with some modifications. Briefly, hydrogel discs were incubated in de-ionized (DI) water for 6 hours with 2X change of DI water, following which they were dried for 24 hours at 37°C. The dried hydrogels were re-swollen in modified simulated body fluid (m-SBF) prepared as detailed by Oyane *et al.* (Oyane *et al.*, 2003). Briefly, 5.402 g NaCl, 0.543 g NaHCO₃, 0.426 g Na₂CO₃, 0.225 g KCl, 0.230 g K₂HPO₄·3H₂O, 0.311g MgCl₂·6H₂O, 17.892 g HEPES (dissolved in 100 ml 0.2N NaOH), 0.293 g CaCl₂ and 10 ml 1M NaOH were added to ultrapure water to obtain a final volume of 1 liter, and the pH was verified to be 7.4.

The m-SBF-re-swollen discs were briefly rinsed in DI water and immersed in a solution of 40 mM Ca²⁺, 24 mM HPO₄²⁻ (pH 5.2, adjusted through dropwise addition of 1

M Tris-HCl at pH 7.5) for 45 minutes on an orbital shaker at 200 rpm. The hydrogels were then briefly rinsed with DI water and immersed in fresh m-SBF for 48 hours at 37°C, with daily exchange of m-SBF. These were then immersed in PBS for 6 hours prior to sterilization. Non-mineralized hydrogels were incubated in phosphate-buffered saline at room temperature after cutting into discs for at least 3 days prior to sterilization.

3.5.3. Scanning Electron Microscopy

Mineralized and non-mineralized hydrogels soaked in PBS were flash frozen using liquid nitrogen and lyophilized for 24 hours. Samples were sputter-coated with Cr (Denton Desk IV Sputter coater) and analyzed using Philips XL30 ESEM to study microstructural topology. Elemental analysis was carried out by using Oxford Energy Dispersive Spectra (EDS) attachment and analyzed using Inca software package.

3.5.4. Measurement of rigidity

Hydrogel samples (mineralized as well as non-mineralized) were equilibrium-swollen in PBS for 3 days prior to compression testing, cut into disks measuring 6 mm in diameter and characterized using Bose ElectroForce 3200 (Bose, Minnesota, USA). Samples were compressed between two parallel plates using a 225 N load cell, with a rate of 0.1 mm/min. The compressive modulus was calculated as the slope of the linear region (between 0-10% strain) of the resulting stress-strain curves. All measurements were carried out in quadruplicates and averaged within each group.

3.5.5. Sterilization of substrates for cell culture

Mineralized and non-mineralized hydrogels, as well as cell culture glass coverslips (Fisherbrand, cat# 1254582) were sterilized in 70% ethanol for 6 hours, and

then immersed in excess of sterile PBS for 4 days with twice daily exchange of PBS to remove residual ethanol. Samples were then incubated in growth medium consisting of high glucose DMEM (GIBCO), 8.97% fetal bovine serum (Hyclone), 1.8 mM L-glutamine (GIBCO) and 50 units/ml penicillin/streptomycin (GIBCO) for 16 hours at 37°C prior to cell seeding.

3.5.6. Cell seeding and imaging

HMSCs (p7071L, Institute for Regenerative Medicine, Texas A & M University) were expanded in growth medium and passaged at 60-70% confluence using 0.25% trypsin-EDTA (Invitrogen). At passage 6 (P6), these cells were seeded on mineralized hydrogels, non-mineralized rigidity matched hydrogels, and cover slips at 5,000 cells/cm² and cultured in growth medium at 37°C, 5% CO₂ for up to 14-21 days. Cells were visualized with bright-field microscopy (Carl Zeiss Axio Observer A1). Cell shape was quantified via shape index (SI) (Schuler *et al.*, 2006) using ImageJ (National Institutes of Health, Bethesda, MD):

$$SI = \frac{4\pi A}{p^2}$$

Where *SI* is the shape index, *A* is the cell area and *p* is the perimeter. For SI, measurements were made from 10 randomly chosen cells, in three different images.

3.5.7. Proliferation analysis

Samples (n=3 per group, per time point) were collected at 7 and 14 days and frozen at -80°C, following which they were lyophilized. Samples were then digested with 1 ml of papain solution (125 µg/ml papain [Worthington Biochemical Corporation], 10

mM L-cysteine [Sigma], 100 mM phosphate and 10 mM EDTA, pH 6.3) for 16 hours at 60°C as in a previous report (Kim *et al.*, 2005). DNA was measured using Quant-IT Picogreen dsDNA Kit (Invitrogen) as previously described (Dadsetan *et al.*, 2008).

3.5.8. Immunofluorescent staining

At 14 and 21 days, samples were fixed in 4% paraformaldehyde (pH 7.4) and immediately stained for osteocalcin (OCN). Briefly, fixed samples were blocked/permeabilized in PBS containing 3% bovine serum albumin and 0.1% Triton-X for 30 minutes. Samples were then incubated with primary antibody (Mouse monoclonal to osteocalcin, [ab13420, Abcam]) diluted 1:100 in blocking solution for 1 hour at room temperature. After washing with PBS for 15 minutes, samples were incubated with secondary antibody (Alexa Fluor 568 goat anti-mouse) and Alexa-Fluor 488 Phalloidin (Invitrogen), diluted 1:250 and 1:100 respectively in blocking solution. Samples were then mounted on glass slides with Vectashield-DAPI (Vector Laboratories) and imaged using a Zeiss Observer A1 microscope equipped with an X-Cite 120 (EXFO) mercury lamp.

3.5.9. Quantitative RT-PCR

Total RNA was extracted from samples (n=3 per group per time point) at 0, 7, and 14 days using TRIzol (Invitrogen) according to the manufacturer's protocol. Per sample, 800 ng of total RNA was reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) reactions were performed using Power SYBR Green PCR Mastermix (Invitrogen) on an Applied Biosciences 7300 Real-Time PCR System (Perkin Elmer/Applied Biosystems).

Target gene expression was normalized to expression of glyceraldehyde ³-phosphate dehydrogenase (GAPDH) as housekeeper. The level of expression of each target gene was calculated as previously reported (Ayala *et al.*, 2011). Primer sequences are provided in Table 3.1.

3.5.10. Alkaline phosphatase assay

Cell-seeded coverslips, mineralized hydrogels, and rigidity matched non-mineralized hydrogels (n=3 per group) were collected for measurement of alkaline phosphatase (ALP) activity at 14 days. Prior to collection, the samples were incubated in serum-free medium for 6 hours in order to eliminate the interference from serum components. Samples were then transferred to a fresh 24 well-plate and incubated in 500 μ l of 0.75M 2-amino-2-methyl 1-propanol (pH 10.3) at room temperature for 5 minutes while shaking at 250 rpm. The supernatant was then collected for coverslip samples; hydrogel samples (both mineralized and non-mineralized) were homogenized in the lysate after incubation and shaking. ALP activity in the supernatant was then measured using ALP substrate kit (Bio-Rad) through the hydrolysis of p-nitrophenylphosphate (p-NPP). Briefly, 30 μ l of the supernatant from the lysate was added to 120 μ l of p-NPP substrate solution (prepared as per the manufacturer's instructions) in a 96 well plate and incubated at room temperature for 5 minutes. The absorbance of each sample was then spectrophotometrically recorded every 20 seconds for 7 minutes at 405 nm using a Beckman Coulter DTX880 Multimode reader. The absorbance was plotted as a function of time for each sample, and the slope was calculated to estimate the ALP activity of the sample. The ALP activity was calculated from the slope and expressed as mmol of p-

nitrophenol formed per liter per minute per ng DNA using the extinction co-efficient of $18.5 \text{ mM}^{-1}\text{cm}^{-1}$ at 405 nm for p-nitrophenol (Henthorn *et al.*, 1988). For each group, ALP activity of a corresponding acellular control was subtracted from the measured ALP activity for the hMSC-seeded samples in order to account for any residual ALP from culture medium.

3.5.11. Dissolution studies

Mineralized hydrogels (n=3) were immersed in 1.5 ml 50 mM Tris-HCl (pH 7.4) at 37°C. Every 24 hours, 200 µl of immersion solution was collected from each sample and replaced with 200 µl of fresh Tris buffer. Calcium concentration in the collected solutions was measured using two reagent calcium set (Pointe Scientific) by o-cresolphthalein complexone according to the manufacturer's instructions. Briefly, the assay solution was prepared as detailed by the manufacturer; 20 µl of sample solution was added to 1 ml of the assay solution. The absorbance of the resulting complex (maroon in color) was measured at 570 nm using a Beckman Coulter DU 730 UV/Vis spectrophotometer. Phosphate concentration was measured using molybdenum yellow (Ducheyne *et al.*, 1993; Heinonen and Lahti, 1981) Briefly, an assay solution was prepared by combining 2 parts of acetone, 1 part 5 N H₂SO₄ and 1 part 10 mM ammonium molybdate. 125 µl of sample solution was added to 1 ml of the assay solution along with 100 µl of 1M citric acid to stabilize the resulting yellow phosphomolybdate complex. The absorbance of the resulting complex was measured spectrophotometrically at 380 nm using a Beckman Coulter DU 730 UV/Vis spectrophotometer.

3.5.12. Effect of soluble Ca^{2+} and PO_4^{3-} on hMSC differentiation

P6 hMSCs were seeded in a tissue culture polystyrene (TCPS) 24-well plate at 5,000 cells/cm² and cultured for up to 7 days in three different medium: (a) growth medium (containing Ca^{2+} concentration of 1.9 mM and PO_4^{3-} concentration of 1 mM), (b) High calcium medium (growth medium containing Ca^{2+} concentration of 3 mM and PO_4^{3-} : 1 mM, prepared by supplementing with 0.22 mg anhydrous CaCl_2 /ml of medium), and (c) high-phosphate medium (growth medium containing Ca^{2+} concentration of 1.9 mM and PO_4^{3-} concentration of 5 mM, prepared by supplementing with 16.2 μl 2M Na_2HPO_4 in DI water and 3.8 μl 2M NaH_2PO_4 in DI water per ml of medium). At 3 and 7 days, total RNA was extracted from samples (n=3 per group per time point) and analyzed for expression of OSX, OCN and Runx2 by using qPCR as detailed earlier.

3.5.13. Statistical analyses

Beyond the biological replicates listed above, experiments were repeated independently at least twice. Statistical analyses were carried out using Graphpad Prism 5.2 (Graphpad Software). In experiments involving two groups, groups were compared using two-tailed Student's t-test ($p < 0.05$). For experiments involving multiple groups across a different time point, comparison was carried out using one way analysis of variance (ANOVA) with Tukey-Kramer post-test to compare individual groups. For experiments involving multiple groups across different time points, comparisons were carried out using two way analysis of variance (ANOVA), with Bonferroni post-test ($p < 0.05$) to compare individual groups.

3.6. Acknowledgements

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Chapter 3, in full, is a reprint of the material as it appears in “Phadke A *et al.* (2012) Mineralized synthetic matrices as an instructive microenvironment for osteogenic differentiation of human mesenchymal stem cells. *Macromol. Biosci.* **12**:1022-1032”. The dissertation author was the primary author on the paper.

3.7. Figures

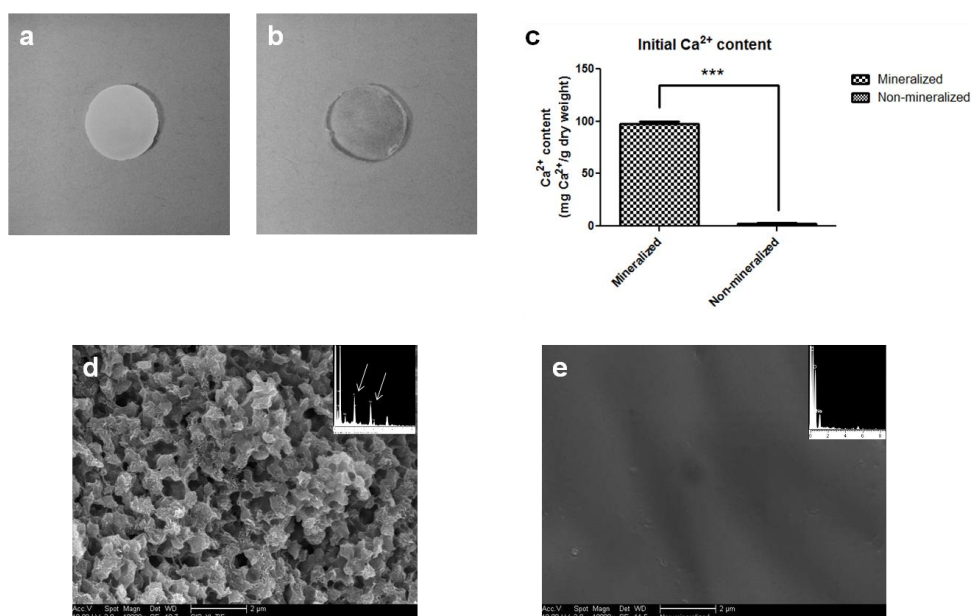


Figure 3.1. Characterization of hydrogels.

Photographs of (a) mineralized and (b) non-mineralized hydrogels. (c) Ca²⁺ content of the mineralized and non-mineralized hydrogels prior to cell culture; asterisks indicate statistical significance (two-tailed, unpaired t-test, ***: $p < 0.001$). Scanning electron microscopy images of (d) mineralized and (e) non-mineralized hydrogels, with corresponding EDS spectra (inset). Arrows indicate spectral peaks corresponding to Ca²⁺ and PO₄³⁻.

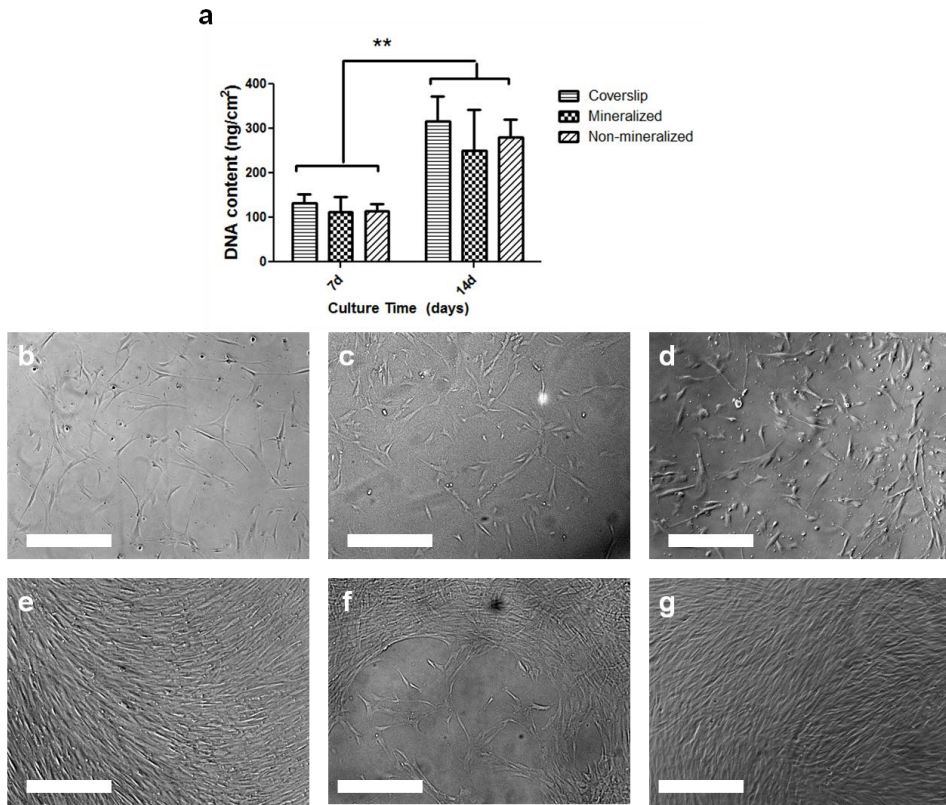


Figure 3.2. Cell proliferation and morphology on various surfaces.

(a) DNA content of the mineralized hydrogels, non-mineralized hydrogels, and coverslips as a function of time. Error bars represent standard error of the mean. Asterisks represent statistically significant differences between the groups indicated ($p < 0.01$). Bright-field images of hMSCs on (b) coverslip (c) mineralized hydrogels, and (d) non-mineralized hydrogels after two days of culture. Bright-field images of hMSCs on (e) coverslip (f) mineralized hydrogels and (g) non-mineralized hydrogels after 14 days of culture. Scale bars represent 500 μm

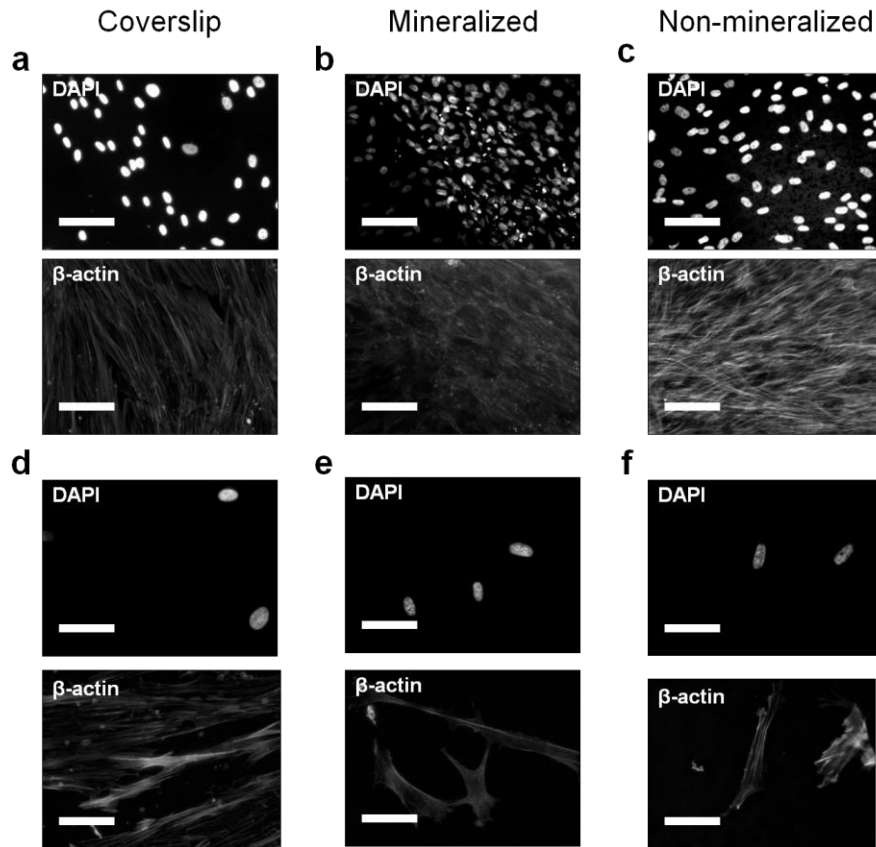


Figure 3.3. Cytoskeletal appearance of hMSCs on various substrates

F-actin staining on (a,d) coverslips, (b,e) mineralized, and (c,f) non-mineralized hydrogels after 14 days of culture. F-actin is stained green and nuclei are stained blue. Scale bars represent (a-c) 100 μm (d-f) 50 μm

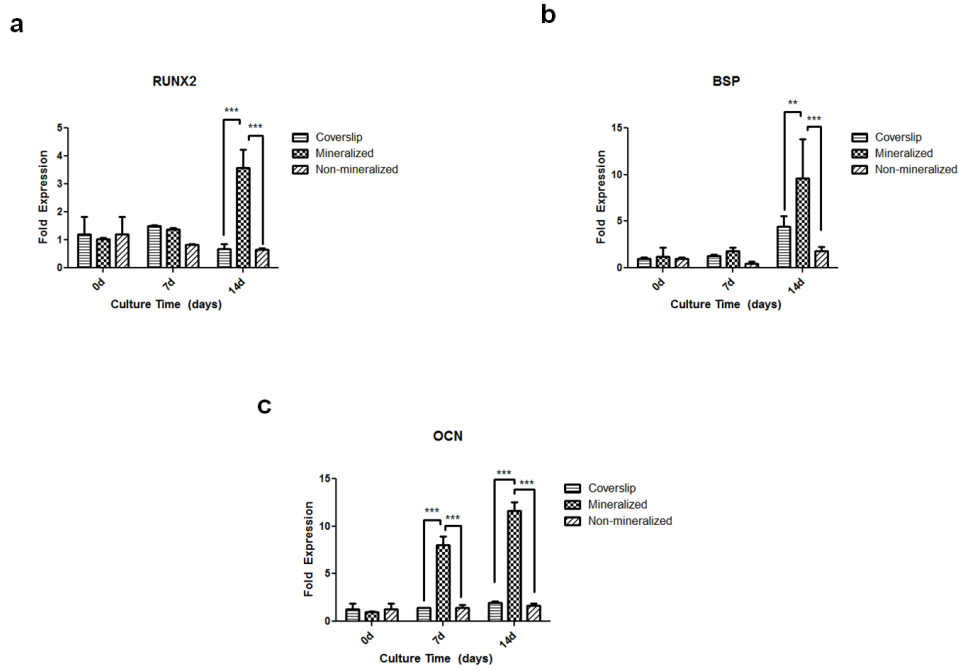


Figure 3.4. Osteogenic gene expression in hMSCs on various substrates.

Expression of (a) RUNX2 (b) BSP and (c) OCN on coverslips, mineralized hydrogels and non-mineralized hydrogels after 7 and 14 days of culture, normalized to expression at day 0 with GAPDH as a housekeeping gene. Error bars represent standard error of the mean (n=3) and asterisks indicate statistically significant differences between the groups compared (**: $p < 0.01$; ***: $p < 0.001$)

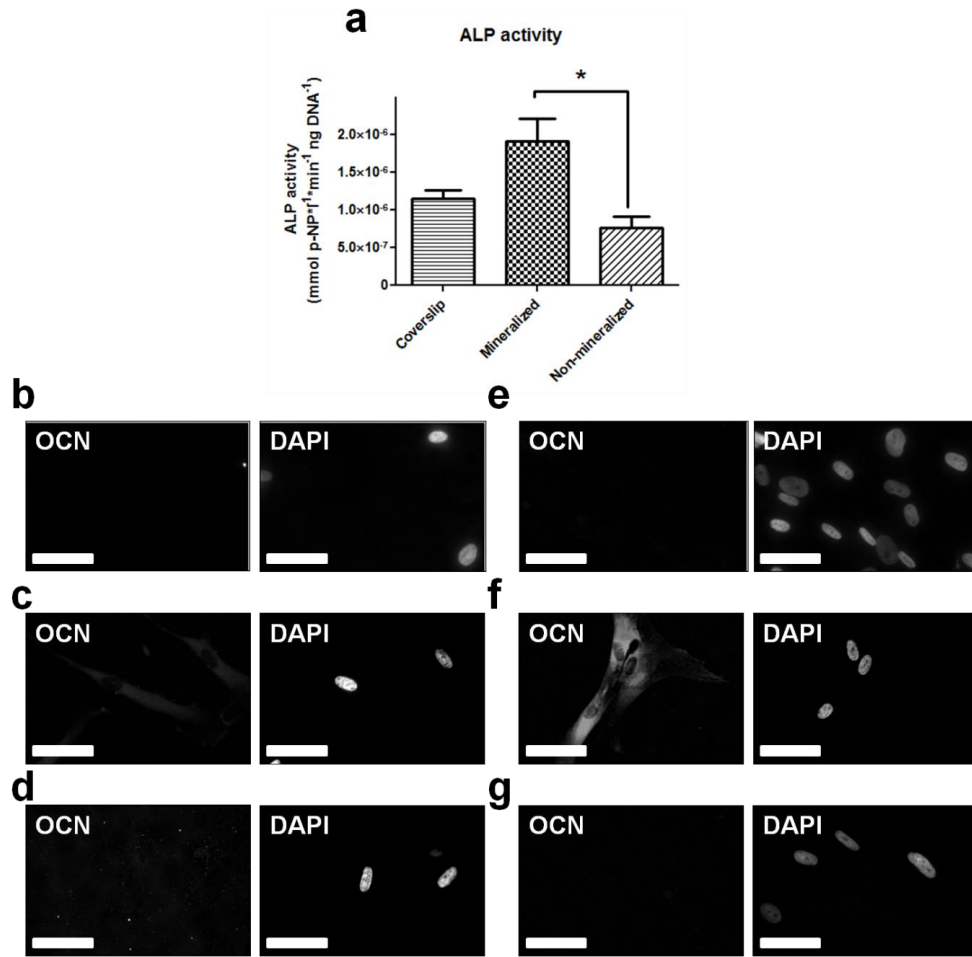


Figure 3.5. Osteogenic differentiation of hMSCs evaluated at the protein level.

(a) Alkaline phosphatase (ALP) activity of hMSCs on coverslips, mineralized hydrogels, and non-mineralized hydrogels after 14 days. Error bars represent standard error of the mean (n=3) and asterisks indicate statistically significant differences between the groups indicated (*: $p < 0.05$). Immunostaining at 14 days for osteocalcin (OCN) in hMSCs seeded on (b) coverslips (c) mineralized hydrogels, and (d) non-mineralized hydrogels. Immunostaining at 21 days for OCN in hMSCs seeded on (e) coverslips, (f) mineralized hydrogels, and (g) non-mineralized hydrogels. Scale bars represent 50 μ m.

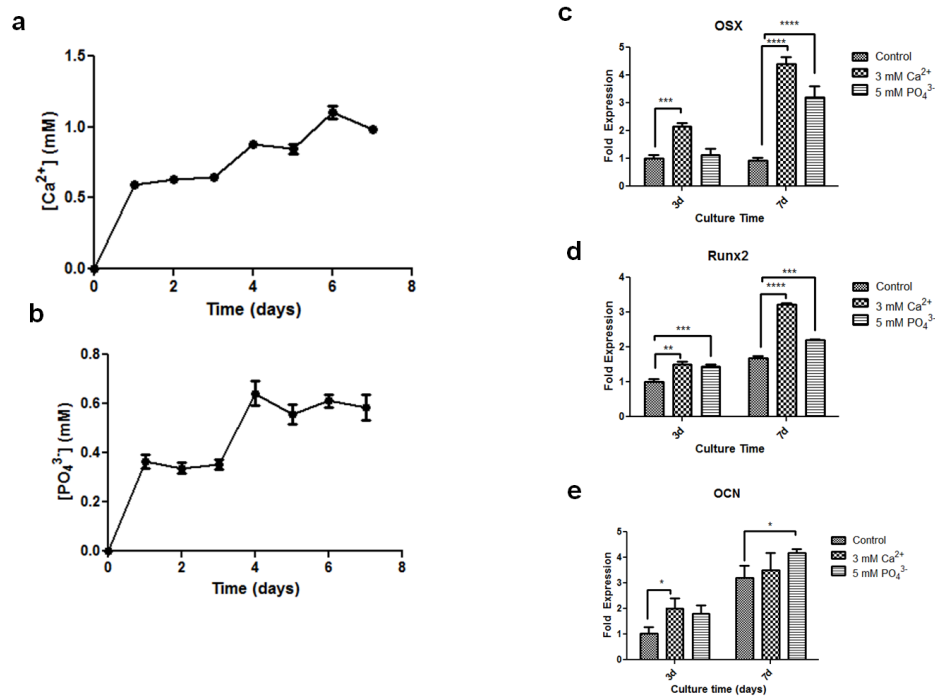


Figure 3.6. Dissolution of the mineralized phase and the role of soluble Ca²⁺ and PO₄³⁻ on osteogenesis.

Release of (a) Ca²⁺ and (b) PO₄³⁻ as a function of time in ion-free Tris (50 mM, pH 7.4). Expression of (c) OSX, (d) Runx2, and (e) OCN in hMSCs cultured in control, high calcium, and high phosphate medium respectively with GAPDH as a housekeeping gene, relative to day 3 samples cultured in control medium. Error bars represent standard error of the mean, asterisks indicate statistically significant differences from control medium (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

3.8. Tables

Table 3.1. List of primers used in qPCR

Gene	Gene (Abbreviation)	Direction	Primer Sequence
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Forward	5' CAT CAA GAA GGT GGT GAA GC 3'
		Reverse	5' GTT GTC ATA CCA GGA AAT GAG C 3'
Osteocalcin	OCN	Forward	5' GAA GCC CAG CGG TGC A 3'
		Reverse	5' CAC TAC CTC GCT GCC CTC C 3'
Runt-related Transcription Factor 2	RUNX2	Forward	5' CCA CCC GGC CGA ACT GGT CC 3'
		Reverse	5' CCT CGT CCG CTC CGG CCC ACA 3'
Bone Sialoprotein	BSP	Forward	5' AAT GAA AAC GAA GAA AGC GAA G 3'
		Reverse	5' ATC ATA GCC ATC GTA GCC TTG T 3'
Osterix	OSX	Forward	5' CAT CTG CCT GGC TCC TTG 3'
		Reverse	5' CAG GGG ACT GGA GCC ATA 3'

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CHAPTER 4: Effect of scaffold microarchitecture on osteogenic differentiation of human mesenchymal stem cells

4.1. Abstract

Design of macroporous synthetic grafts that can promote infiltration of cells, their differentiation, and synthesis of tissue-specific extracellular matrix is a key determinant for *in vivo* bone tissue regeneration and repair. In this study, we investigated the effect of microarchitecture of the scaffold on osteogenic differentiation of human mesenchymal stem cells (hMSCs). Poly(ethylene glycol) diacrylate-co-N-acryloyl 6-aminocaproic acid cryogel scaffolds were fabricated to have either a pore network consisting of cellular, randomly oriented pores (termed ‘spongy’) or a pore network consisting of lamellar columns (termed ‘columnar’), with both cryogel types showing a similar overall porosity. Both types of cryogels supported similar levels of cell viability and proliferation of hMSCs *in vitro*. However, spongy cryogels promoted osteogenic differentiation to a greater extent than their columnar counterparts, as evidenced by increased alkaline phosphatase activity and osteoblastic gene expression. Leveraging upon our previous work, we further evaluated the ability of these synthetic scaffolds in conjunction with mineralization, to promote ectopic bone formation upon subcutaneous implantation in nude rats. Mineralized spongy and columnar cryogel scaffolds, both in the absence and presence of exogenous hMSCs, promoted ectopic bone formation at 9 weeks *in vivo*. No such bone formation was observed in non-mineralized cryogel scaffolds, with extensive host cell infiltration and vascularization in columnar cryogel scaffolds, and negligible

infiltration into spongy cryogel scaffolds. Our results thus present a novel method to tune the microarchitecture of porous polymeric scaffolds, in addition to suggesting their efficacy as synthetic bone grafts.

4.2. Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitors that have been isolated from a variety of adult tissues and are capable of self-renewal as well as differentiation into lineages specific to bone, fat, and cartilage under specific culture conditions (Phadke *et al.*, 2010a). Osteogenic differentiation of MSCs is characterized by increased alkaline phosphatase (ALP) activity, expression of bone-specific markers (including the transcription factor Runx2, as well as matrix proteins such as osteocalcin, bone sialoprotein and osteopontin), and the deposition of calcified matrix (Hwang *et al.*, 2007; Hwang *et al.*, 2009a; Jaiswal *et al.*, 1997). Indeed, the differentiation of MSCs into the osteoblastic lineage has been touted as a promising strategy for *in vivo* regeneration and repair of damaged bone tissue (Bruder *et al.*, 1998; Cowan *et al.*, 2004; Kadiyala *et al.*, 1997). For the effective therapeutic application of MSCs in bone repair and regeneration, it is necessary to utilize grafts that support osteogenic differentiation of progenitor/stem cells and vascularization of the implant. Emerging studies clearly indicate the role of cues arising from extracellular matrix on directing osteogenic commitment of stem cells. In particular, the structural, topographical, physical, and chemical characteristics of the matrix play an important role in determining osteogenic differentiation of progenitor cells and bone tissue formation (Ayala *et al.*, 2011; Benoit *et al.*, 2008; Curran *et al.*, 2006; Dalby *et al.*, 2007; Engler *et al.*, 2006; Yuan *et al.*, 2010).

The microarchitecture of the scaffold plays an important role in osteogenic differentiation of stem cells in a 3-dimensional environment, as differences in microstructure can influence cell infiltration, nutrient transport, extracellular matrix accumulation, and vascularization (Hutmacher *et al.*, 2007; Karageorgiou and Kaplan, 2005; Zeltinger *et al.*, 2001). Given these effects of scaffold pore microarchitecture, it is important to gain an understanding of its effect on the osteogenesis of human MSCs (hMSCs). The hierarchical structure of native bone tissue suggests that the pore architecture could play a key role in osteogenic differentiation. For instance, trabecular and cortical bone possess significantly different pore architecture. Trabecular bone is highly porous (70-90% porosity) with isotropic, cellular pore architecture (Galante *et al.*, 1970), while cortical bone is much denser than trabecular bone with > 15% porosity, with a pore network of oriented, columnar pores (known as a Haversian system) (Schaffler and Burr, 1988; Wang and Ni, 2003).

Previous studies have demonstrated the effect of pore structure, pore size, and scaffold porosity on the in-growth of bone tissue, as well as ectopic bone induction *in vivo* (Gauthier *et al.*, 1998; Kim *et al.*, 2010; Kühne *et al.*, 1994; Mastrogiacomo *et al.*, 2006; Tsuruga *et al.*, 1997; von Doernberg *et al.*, 2006). Similarly, studies have also shown the effect of pore architecture of ceramic scaffolds on the activity of murine osteoblasts *in vitro* (Fu *et al.*, 2009). However, the effect of pore architecture (i.e. pore shape and morphology of pore network) on *in vitro* osteogenic differentiation of hMSCs is unclear, though previous studies have elucidated the effect of pore size and porosity on osteogenesis (Gomes *et al.*, 2006; Kasten *et al.*, 2008). To this end, we have investigated

the role of the microarchitecture of scaffolds on the osteogenesis of hMSCs using cryogels with distinctly different pore architecture, while having similar porosity. Specifically, we have utilized poly(ethylene glycol) -*co*-N-acryloyl 6-aminocaproic acid (PEGDA-*co*-A6ACA) cryogels with two types of pore microarchitecture: spongy pore architecture, consisting of an isotropic network of cellular pores and columnar pore architecture, having a directional network of plate-like columnar pores. The cryogels with different architectures were fabricated by using a new method involving principles of cryogelation.

4.3. Results

4.3.1. Development and characterization of cryogels with varying pore architecture

Previously, we have shown that the internal microstructure of cryogels can be controlled through various cryogelation parameters such as gelation temperature, degree of supercooling, and kinetics of polymerization (Hwang *et al.*, 2009). In this study, we have harnessed the ability to control the structure of the ice network to develop cryogels with unique internal pore architectures without altering their chemistry or overall porosity (Figure 4.1). Indeed, controlling the ice network structure yielded cryogels with two types of distinct pore microstructure, referred to as ‘spongy’ and ‘columnar’, (Figure 4.1,2). As shown in Figure 4.2a-b, a directional cooling front during cryogelation promoted the growth of ice columns perpendicular to the ice-polymer solution interface, and therefore cryogels having a lamellar columnar structure with a larger pore size of approximately 50-60 μm in the dried state (corresponding to $\sim$ 100-150 μm in the swollen

state, as estimated through bright field microscopy) were synthesized. The pore structure consisted of several oriented lamellae of columns, connected through smaller pores (subsequently referred to as ‘columnar pore structure’). On the other hand, their counterparts synthesized without preferential nucleation sites (in spongy molds) led to a macroporous network consisting of more randomly oriented, interconnected cellular pores measuring approximately 20-30 μm in diameter in the dried state (corresponding to 50-60 μm in the swollen state, as estimated via bright field microscopy) throughout the constructs as shown in Figure 4.2c-d; the pore network lacked any particular orientation and will subsequently be referred to as ‘spongy’ pore structure. Between the two structures, the spongy cryogels showed a substantially higher pore area ($0.37 \pm 0.18 \text{ m}^2/\text{g}$) than columnar cryogels ($0.17 \pm 0.05 \text{ m}^2/\text{g}$) (Table 4.1). Interestingly, despite the vast difference in pore shape and size, mercury intrusion porosimetry suggested similar porosity between the spongy and columnar cryogels; spongy cryogels had a porosity of $70.0 \pm 0.4\%$ while columnar cryogels had a porosity of $70 \pm 5\%$. (Table 4.1) Porosity measurements for columnar cryogels were further supported through analysis of the micro-CT reconstructions which suggested a porosity of 67% for columnar constructs; the small size of pores in the lyophilized spongy cryogels coupled with insufficient resolution of the scanner did not allow for their accurate porosity quantification through micro-CT.

4.3.2. Effect of pore structure on osteogenic differentiation of hMSCs *in vitro*.

Following 24 hours of culture in growth medium, cells showed similar viability ($>90\%$) in both spongy and columnar cryogels (Figure 3a-b). A notable difference

between the two cryogels, however, was the difference in morphology of the cells within the cryogels. Cells cultured in spongy cryogels showed a more spread morphology, compared to cells seeded in columnar cryogels which formed small cellular aggregates along the pore walls (Figure 4.3a-b). DNA quantification at 7 and 21 days demonstrated proliferation of cells between 0 and 7 days (approximately 1.5-fold), with both cryogels showing similar DNA content (Figure 4.3c). However, no significant proliferation was observed between 7 and 21 days. Spongy cryogels showed a slight decrease in cell content with culture time, although the decrease was not statistically significant. The similar DNA content after 21 days suggests similar cell proliferation between the spongy and columnar cryogels.

Gene expression analyses suggested that both the cryogels supported osteogenic differentiation of the hMSCs compared to day 0 controls, as evidenced by the upregulation of the osteogenic markers Runx2, OCN, and OPN throughout the culture period (Figure 4.4a-c). However, there were significant differences in the temporal expression levels depending upon the type of cryogel microstructure. Specifically, cells in spongy cryogels showed significantly higher upregulation of Runx2 at 4 days of culture, OPN at 14 days of culture and OCN at 21 days of culture, when compared to columnar cryogels. This suggests that the spongy cryogels promoted osteogenic differentiation at a more rapid pace than the columnar cryogels.

The extent of osteogenesis in the cryogels was additionally measured by quantifying the activity of alkaline phosphatase, as well as the cell-mediated calcification. Indeed, spongy cryogels showed substantially higher ALP activity than columnar

cryogels at all time points up to 21 days of culture (Figure 4.4d). Additionally, we observed that spongy cryogels showed higher calcium content (Figure 4.4E), although the difference was not statistically significant. Given the similar DNA content of spongy and columnar cryogels throughout the study, this suggests that the spongy cryogels were more conducive to osteogenic differentiation than the columnar cryogels.

Immunofluorescent staining also revealed the presence of OCN in hMSCs cultured in both spongy and columnar cryogels, providing further evidence that these cryogels supported osteogenic differentiation (Figure 4.4f,g).

4.3.3. *In vivo* tissue formation

Previously, we have demonstrated the ability of mineralized PEG-A6ACA hydrogels to induce osteogenic differentiation of hMSCs, even in the absence of osteogenic-inducing soluble factors and growth factors employing a 2-dimensional culture condition (Phadke *et al.*, 2012). Thus, we next studied the *in vivo* subcutaneous response to mineralized and non-mineralized cryogels in a nude rat model. First, the mineralization of the cryogels was confirmed through scanning electron microscopy and elemental analysis (Figure 4.5). Elemental analysis revealed a Ca/P ratio of 1.7, suggesting the presence of an apatite-like mineralized phase. We then examined the effect of mineralization on hMSC viability *in vitro* by seeding hMSCs on scaffolds mineralized zero, one and two times respectively, and examining their viability after four days of culture- excessive mineralization of scaffolds has been demonstrated to negatively affect cell viability possibly due to unfavorable alkaline shifts in pH (Linhart *et al.*, 2001). Indeed, an increase in cell death was observed with increased mineralization

(Figure 4.S1a); a sizeable drop-off in viability was observed from once-mineralized to twice-mineralized scaffolds respectively, while the drop-off in viability from the non-mineralized to the once-mineralized scaffolds was observed to be relatively minor. Moreover, the twice-mineralized scaffolds were found to effect a dramatic increase in pH (from an initial pH of 7 to approximately pH 9) when soaked in DI water (Figure 4.S1b). As a result, we decided to proceed with the once-mineralized scaffolds for *in vivo* studies.

Next, the ability of the implanted cryogels to promote bone tissue formation was evaluated by using a number of different acellular and cellular cryogels. These include (i) acellular columnar cryogels (non-mineralized), (ii) acellular spongy cryogels (non-mineralized), (iii) acellular columnar cryogels (mineralized), (iv) acellular spongy cryogels (mineralized), (v) hMSC-seeded columnar cryogels (mineralized) and (vi) hMSC-seeded spongy cryogels (mineralized). No evidence of bone formation was observed in radiographs at 2 weeks; however, beginning at 4 weeks, hard tissue was visible in radiographs of all implants and persisted at 6 and 9 weeks (Figure 4.6a,b). After 9 weeks of implantation, all mineralized cryogels (groups iii-vi) showed evidence of hard tissue formation, irrespective of pore structure or seeding with hMSCs prior to implantation. Mineralized cryogels showed the formation of peripheral hard tissue, as observed through micro-CT (Figure 4.6c). Gross examination post-excision from implantation sites also revealed infiltration of host cells into the mineralized cryogels (Figure 4.7). Histological analysis revealed the formation of bone-like tissue in the hard tissue layer, accompanied by the in-growth of host vasculature into the scaffold, suggesting that the mineralization of the cryogels played a crucial role in the

vascularization and bone formation (Figure 4.7). Additionally, the hard tissue layer showed the presence of OCN as evidenced by immunohistochemical staining, further suggesting that the mineralized cryogels promoted bone formation *in vivo* (Figure 4.8). It is important to note that the presence of the mineral layer was expected to reduce the effective size of the pores. Mercury intrusion porosimetry revealed that mineralization only reduced average pore diameter by approximately 2 μm , suggesting that mineralization was unlikely to restrict access into construct interior through pore closure. Moreover, in order to address whether the bone-like morphology of the tissue was due to appositional mineralization of the scaffold *in vivo* without bone formation, we examined the histology of mineralized cryogels allowed to mineralize *in vitro* under physiological conditions (Figure 4.S2). The difference in histology of the mineralized phase of these samples with the morphology of the hard tissue *in vivo* supported our conclusion that the hard tissue *in vivo* was in fact, bone.

Non-mineralized cryogels (groups i-ii), on the other hand, showed a dramatically different response from their mineralized counterparts. Unlike the mineralized cryogels, no hard tissue formation was observed even at 9 weeks of implantation for non-mineralized cryogels, results supported by the lack of hard tissue detection in micro-CT and digital X-rays. Moreover, unlike the mineralized cryogels, the pore architecture was seen to play a major role in host cell infiltration into the non-mineralized cryogels. Non-mineralized cryogels with spongy pore architecture showed negligible host cell infiltration, as observed through gross examination as well as histological analyses (Figure 4.7). Non-mineralized columnar cryogels on the other hand, showed host cell

infiltration, but with abundant fibrous tissue and extensive vascularization in the scaffold interior, without any bone information as indicated by the lack of OCN staining (Figure 4.7,8). The histology, lack of hard tissue formation and immunohistochemical analyses suggested that the non-mineralized columnar cryogels did not promote bone formation *in vivo*.

4.4. Discussion

During freezing of aqueous solutions, the morphology of the ice network is governed by the directionality of the cooling front and the solid-liquid interface between the solid ice and liquid aqueous solution, both of which control the directionality of the ice network (Zhang *et al.*, 2005). We have previously demonstrated the ability to synthesize macroporous poly(ethylene glycol) cryogel scaffolds (Hwang *et al.*, 2010; Hwang *et al.*, 2009). In this study, we manipulated the directionality of ice crystal formation by controlling the directionality of the cooling front and the solid-liquid interface (between the solid ice and the liquid polymer precursor solution) in order to vary the internal pore architecture of the cryogel scaffolds. To create the directionality of internal pore structures, we have introduced a thin ice layer at the bottom of the mold to provide preferential nucleation sites, allowing for columnar ice dendrite growth without changing the precursor composition. The underlying ice layer provides a more drastic temperature gradient within the polymerizing solution from top to the bottom in the columnar molds than their spongy counterparts, which have the exact same precursor composition but a more uniform, isotropic temperature gradient because they lack the pre-formed ice layer. In the case of the columnar cryogels, the presence of an ice layer in

the bottom of the mold prior to polymerization leads to a cooling front that advances the growth of ice crystal perpendicular to the solid-liquid interfaces (uni-directionally upward from the bottom of the mold). This ice bed provides a template from which dendritic ice crystals grow in a lamellar fashion. This lamellar, oriented ice network provides directionally oriented lamellar, columnar pores upon thawing of the network. Spongy cryogels on the hand, were prepared using isotropic cooling that led to randomly oriented, cellular pores as observed in our previous work which made use of isotropic cooling to form cryogels with randomly oriented, cellular pores (Hwang *et al.*, 2010). This led to the formation of an isotropic ice network that did not favor any particular orientation, and therefore resulted in an isotropic pore network. Such manipulation of ice network formation by tuning the directionality of the cooling front has been previously used to tune the ceramic scaffold microarchitecture (Dewille, 2008; Wegst *et al.*, 2010; Yunoki *et al.*, 2006). Indeed, a previous study reported the simultaneous presence of a columnar-like pore zone and spongy-like cellular pore zone during the freeze-casting of hydroxyapatite through manipulation of the freezing front morphology and the freezing kinetics (Dewille *et al.*, 2006). Our work however, demonstrates for the first time, the application of this technique in the formation of synthetic polymeric scaffolds with distinctly different microarchitecture mimicking different structural attributes of bone tissues. Such scaffold could be a tool to gain insight into the effect of pore microarchitecture on cellular functions *in vitro* and *in vivo*.

Our observation that despite having the same porosity, spongy cryogels promoted *in vitro* osteogenic differentiation of hMSCs shows the role of scaffold microarchitecture

on cellular functions. This is consistent with a previous study, which shows a similar effect of pore architecture of freeze-casted hydroxyapatite scaffolds on murine osteoblast activity (Fu *et al.*, 2009); in that study, isotropic, cellular interconnected pore structures (similar to the spongy pore architecture in our study) promoted ALP activity of the cells relative to lamellar, plate-like pores (similar to the columnar pore architecture in our study) despite a similar porosity between the two scaffold types. Despite the differences between the study by Fu *et al.* and our study vis-à-vis the material used as well as the cell types, their data concurs with our findings in that scaffolds with isotropic, cellular pore architecture are superior to those with oriented, lamellar pore structure in supporting early osteogenic differentiation of progenitor cells. Moreover, the study by Fu *et al.* utilized spongy-like pore architecture with large pore size and columnar pore architecture with smaller pore size, while our study utilizes spongy pore architecture with smaller pore size and columnar pore architecture with larger pore size. That the spongy pore morphology is superior in both studies despite this opposite trend in pore sizes suggests that the pore morphology plays a dominant role behind the enhanced osteogenesis in the spongy cryogels, while the difference in pore size is unlikely to be a significant contributor to the observed effect of microarchitecture on osteogenic differentiation *in vitro*.

The enhanced osteogenic differentiation of hMSCs in spongy cryogels could have been influenced by increased mass transport due to differences in interconnectivity between the pores. In fact, Uebersax *et al.* have previously demonstrated that pore interconnectivity can indeed influence osteogenic differentiation *in vitro* (Uebersax *et al.*,

2006). In our study, the columnar scaffolds could have had fewer interconnections between the macropores owing to the plate-like lamellar pore structure; along with affecting nutrient transport throughout the scaffold, pore interconnectivity can also influence transport of cell-secreted factors throughout the scaffold, with more optimal transport expected in the case of the spongy scaffolds. Cell-cell communication via cell secreted soluble factors has been shown to play a significant role in the osteogenesis of MSCs (Birmingham *et al.*, 2012). In addition to interconnectivity, tortuosity has also been proposed to affect osteogenesis (Kim *et al.*, 2010); it is highly possible that the isotropic, dendritic pore structure of spongy scaffolds possessed a dramatically different tortuosity from the plate-like pores in the columnar scaffolds which could have influenced the osteogenic differentiation of the hMSCs.

Another possible factor is the cell shape; hMSCs seeded in spongy cryogels appeared to assume spread morphology over the spongy cryogel pore surface, while hMSCs in columnar cryogels formed aggregates with roughly a spherical morphology in the pores without significant spreading. This could have been due to the fact that although the porosities were very similar between the two scaffold types, the pore surface area was higher (almost 2-fold) in the spongy cryogels than the columnar cryogels, thereby allowing for increased available area for spreading of the cells in spongy cryogels following their seeding. As spread morphology has been previously demonstrated to favor osteogenesis of hMSCs, this could be another factor favoring osteogenic differentiation in the spongy cryogels (McBeath *et al.*, 2004).

The formation of ectopic bone tissue in all mineralized cryogels, irrespective of pore architecture and the presence/absence of hMSCs suggest that the presence of the mineralized phase led to recruitment of endogenous progenitors from the surrounding host tissue. In addition to osteoinduction, the presence of a mineralized phase also promoted vascularization of the implants, as evidenced by the gross appearance of the excised implants, which suggested vascularization of mineralized acellular spongy scaffolds and the lack of vascularization in acellular non-mineralized spongy scaffolds. This is in agreement with a previous study that showed improved angiogenesis in polymer-ceramic composites when compared to pure polymeric scaffolds (Gerhardt *et al.*, 2011).

The mineralized matrix-mediated osteoinduction observed in this study is highly intriguing as ectopic bone formation in calcium phosphate-based scaffolds such as β -TCP and HA in the absence of any exogenously added cells has been conclusively established in large animal models such as dogs, pigs, sheep and primates, but most studies report that these materials are unable to induce bone formation in small animals such as rats and mice (Yang *et al.*, 1996; Yuan *et al.*, 2006). However, synthetic material-induced bone formation in the absence of exogenous cells or biologics as observed in our study, while rare, is not unprecedented in rodent models (Barradas *et al.*, 2012; Cheng *et al.*, 2012; Pek *et al.*, 2008; Selye *et al.*, 1960; Seyedjafari *et al.*, 2010). A previous study noted the formation of mature bone in glass diaphragms implanted subcutaneously in rats (Selye *et al.*, 1960). Several of these studies employ composite matrices wherein calcium phosphates are either physically mixed with, or coated on an organic substrate and can

induce ectopic bone formation (Pek *et al.*, 2008; Seyedjafari *et al.*, 2010) and bone defect healing (Kawai *et al.*, 2008) in rodents. It is possible that the mineralized A6ACA-PEGDA matrices in our study provided a more effective mimic of bone tissue through their composite, biomineralized nature. In fact, work by Osathanon *et al.* has demonstrated that biomineralized fibrin matrices promoted enhanced osteoblastic activity compared to their non-mineralized counter parts physically embedded with minerals by physical mixing, providing further support of the influence of the biomineralization process on the osteoinductivity of materials (Osathanon *et al.*, 2008).

The ability of biomineralized matrices to promote ectopic bone tissue formation could be attributed to a variety of physicochemical cues arising from the mineralized matrix such as the topographical cues arising from the minerals, as well as chemical cues arising from the dynamic dissolution-precipitation of the mineralized phase (Chai *et al.*, 2012). The dissolution-precipitation of mineralized materials lead to increased local Ca^{2+} and PO_4^{3-} concentrations, which have been demonstrated to promote osteogenic differentiation of progenitor cells *in vitro* (Chai *et al.*, 2012; Chai *et al.*, 2010; Phadke *et al.*, 2012) and could play a role in osteoinduction by these materials *in vivo* (Chai *et al.*, 2010; Le Nihouannen *et al.*, 2005; Müller *et al.*, 2008; Phadke *et al.*, 2012). Ionic dissolution products from calcium-containing ceramics such as bioactive glasses have also been linked to a pro-angiogenic effect of these materials *in vitro* and *in vivo* (Gorustovich *et al.*, 2010). Beyond regulating the local concentration of Ca^{2+} and PO_4^{3-} ions, the dissolution-precipitation of the mineral phase can also sequester pro-osteogenic and pro-angiogenic growth factors such as bone morphogenetic proteins

(BMPs) and vascular endothelial growth factors (VEGFs), thereby potentially leading to the observed osteoinduction and vascularization (Lee et al., 2011). Additionally, genetic factors relating to intra-species differences between different strains of the same species can influence bone formation. A recent study has demonstrated that in mice, the ability of calcium phosphates to induce ectopic bone formation was strain-dependent with specific strains showing far superior ectopic bone formation ability when compared to other strains (Barradas *et al.*, 2012). It is possible that our results were influenced by specific genetic factors arising from the use of the athymic *nu/nu* strain of rats utilized in our study.

The differential host cell infiltration into the non-mineralized spongy and columnar cryogels could be attributed to the difference in pore size between the cryogels. A recent study demonstrated that a pore size of 100 μm -150 μm is more conducive to host cell infiltration into macroporous hydrogel scaffolds than a pore size of 25 μm -50 μm (Chiu *et al.*, 2011); similarly in our study, the large pore size of the columnar PEGDA-*co*-A6ACA cryogels (100-150 μm) could be more conducive to vascular in-growth than the relatively smaller pore size (50-60 μm) of the spongy cryogels, resulting in extensive host cell infiltration and vascularization of the former but not the latter. However, the bioactivity imparted by the presence of the mineral layer was sufficient to overcome this issue, as evidenced by the vascularization and host cell infiltration into the mineralized spongy cryogels, in stark contrast with the lack of host cell penetration observed in the non-mineralized spongy cryogels.

4.5. Conclusion

We have demonstrated the ability to control pore architecture in macroporous cryogels during cryogelation by controlling the directionality of the cooling front, obtaining cryogels with a lamellar, columnar pore structure as well as spongy pore architecture. Further, we have demonstrated that in macroporous cryogel scaffolds, cellular, isotropic pore architecture promotes early osteogenic differentiation of hMSCs to a greater extent than lamellar, oriented pore structure. *In vivo*, the presence of a mineral phase in these cryogels promoted ectopic bone formation irrespective of pore architecture, although pore microarchitecture did significantly influence host cell infiltration and vascularization in the absence of a mineral phase.

4.6. Acknowledgements

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Chapter 4, in full, is a reprint of the material as it appears in “Phadke A, Hwang Y *et al.* (2012) Effect of scaffold microarchitecture on osteogenic differentiation of human mesenchymal stem cells. *Eur. Cells Mater.* (in revision)”

4.7. Experimental section

4.7.1. Monomer synthesis

N-acryloyl 6-aminocaproic acid (A6ACA) and poly(ethylene glycol)-diacrylate (M_n : 3.4kDa) (PEGDA3.4K) were synthesized as previously described (Ayala *et al.*, 2011; Zhang *et al.*, 2009).

4.7.2. Cryogel synthesis

Cryogels were prepared in cylindrical polypropylene molds measuring 3 mm in diameter. For columnar cryogels, 40 μ l of DI H₂O was added to the molds and chilled at -20°C prior to cryogelation to create a thin ice layer at the bottom of the mold (referred to as ‘columnar molds’). For cryogels with spongy pore structure, molds were chilled at -20°C without DI H₂O (referred to as ‘spongy’ molds). Both spongy and columnar molds were chilled in a covered dry polystyrene petri dish.

A solution of 9.25% w/v A6ACA, 20% w/v PEGDA3.4K was prepared in 0.5M sodium hydroxide and chilled to 4°C for 1 hour. To this chilled precursor solution, 0.15% tetramethylethylenediamine and 0.5% ammonium persulfate were added to initiate polymerization. Immediately, 60 μ l of the resulting solution was added to spongy and columnar molds respectively, and placed inside covered dry polystyrene petri dishes. This setup was then placed at -20°C and allowed to polymerize for 24 hours. Following polymerization, both spongy and columnar cryogels were removed from molds, thawed at room temperature and immersed in 1X phosphate-buffered saline (PBS).

4.7.3. Micro-computed tomography

The cryogels were soaked in a 10% solution of FeCl_3 in DI H_2O for 2 hours, frozen at -80°C , and then lyophilized. The function of the FeCl_3 was to provide Fe^{3+} ions to bind to the anionic A6ACA moieties, causing iron salt formation on the internal surface of the cryogels upon lyophilization. The FeCl_3 salts on the internal pore layer served to increase contrast during radiographic imaging. The 3-dimensional internal pore structure of the cryogels was non-invasively imaged using the SkyScan 1076 High Resolution In-Vivo Micro-Computed Tomography Scanner (Skycan, Belgium), at $9\text{ }\mu\text{m}/\text{pixel}$ resolution. Scans were reconstructed from projections using NRecon software (SkyScan, Belgium) and converted to 3-dimensional objects using DataViewer software (SkyScan, Belgium). Additionally, porosity measurements were carried out via CTAn software (SkyScan, Belgium).

4.7.4. Scanning electron microscopy

The microstructures of PEGDA-co-A6ACA cryogels were examined using scanning electron microscopy (SEM, Philips XL30 ESEM). Briefly, the samples were dehydrated in 50%, 75%, and 100% ethanol and dried using a critical point dryer (Tousimis AutoSamdri 815). After samples were completely dried, they were Iridium-coated using a sputter coater (Emitech K575X Sputter Coater) for 8 seconds prior to SEM imaging.

4.7.5. Porosity

A mercury intrusion porosimeter (MIP) (Micromeritics AutoPore IV9500, Oak Ridge, TN, USA) was used to determine the internal pore size distribution, pore surface

area, and porosity. Briefly, the samples were serially dehydrated in 50%, 75%, and 100% ethanol. They were then dried using a critical point dryer (Tousimis AutoSamdri 815) in which they were subjected to a pressure cycle starting at approximately 0.5 psia, increasing to 60000 psia. Based on the amount of intrusion of mercury into samples via their internal pore structures, the analysis was performed using AutoPore IV9500 v1.07 software. A total of 0.1 g of each sample was used for the measurement.

4.7.6. Cell culture

HMSCs (Center for Regenerative Medicine, Texas A & M University) were expanded at 37°C, 5% CO₂ in growth medium consisting of high-glucose DMEM (Gibco), 8.97% fetal bovine serum (Hyclone), 1.8 mM L-glutamine (Gibco), and passaged at 70% confluence using 0.25% trypsin-EDTA (Invitrogen). Cells were utilized at passage 6 for seeding within the cryogel scaffolds.

4.7.7. Cryogel sterilization

Following synthesis, cryogels were immersed in 1X PBS for 24 hours (with 2X change of solution) to remove the unreacted materials. Cryogels were then sterilized by immersion in 70% ethanol for 12 hours and washed in 1X sterile PBS for at least 3 days (with at least 2X daily change of solution) to remove residual ethanol. Cryogels were immersed in growth medium at 37°C, 5% CO₂ for 18 hours prior to cell seeding.

4.7.8. Cell seeding

At time of seeding, cryogels were dried under sterile conditions for 105 minutes, resulting in an approximately 50% and 40% loss of weight through evaporation for spongy and columnar cryogels, respectively. Cryogels were then seeded at a density of

8×10^5 cells/construct. Briefly, hMSCs were suspended in growth medium at 1.33×10^7 cells/ml. 60 μ l of this suspension was then seeded on top of each construct at various spots. The seeded constructs were then incubated in the absence of medium at 37°C, 5% CO₂ for 3 hours to allow for cell attachment. Following this, cell-laden cryogels were incubated in growth medium for 24 hours at 37°C, 5% CO₂. Subsequently, cryogels were cultured for up to 21 days in osteogenic medium consisting of high-glucose DMEM (Gibco), 10% FBS (Hyclone), 10 mM β -glycerolphosphate (Calbiochem), 100 nM dexamethasone (Sigma), 10 μ g/ml ascorbic acid-2-phosphate (Sigma), and 50 units/ml penicillin-streptomycin (Gibco). Medium was changed every 48 hours and samples were collected for analysis as detailed below.

4.7.9. Cell viability

Following 24 hours after culture in growth medium post-seeding, cryogels were analyzed for viability of the seeded cells. The cryogels were cut into slices, stained with a Live/Dead Viability/Cytotoxicity Kit (Invitrogen) and subsequently visualized using via fluorescent microscopy to distinguish live cells (stained green by calcein-AM) from dead cells (stained red by ethidium homodimer). Viability was estimated by comparing the number of live cells to the total number of cells.

4.7.10. Quantitative PCR

Cell-laden cryogels (constructs) were analyzed for expression of osteogenic markers as a function of culture time at 4, 7, 14, and 21 days, and compared to d0 hMSCs collected at the time of seeding. Constructs (n=3 per group, per time point) were homogenized in TRIzol (Invitrogen); RNA was extracted from this homogenate

according to the manufacturer's instructions. 800 ng of RNA was reverse-transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The resultant cDNA was then analyzed for expression of osteogenic markers — Runx2, osteocalcin (OCN), and osteopontin (OPN) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Expression at each time point was normalized to day 0 and expressed as fold change thereof. Real-time PCR reactions were run on a Model 7300 Real-time PCR Cyclor (Applied Biosystems), using Power SYBR I Mastermix (Applied Biosystems). Expression level of various genes was calculated as previously described (Varghese *et al.*, 2010). RT-PCR primers used are listed in Table 4.2.

4.7.11. Alkaline phosphatase activity

Spongy and columnar cryogels were assayed for activity of alkaline phosphatase (ALP) using enzymatic dephosphorylation of para-nitrophenolphosphate (p-NPP) to para-nitrophenol (n-NP) at various time points (4, 7, 14 and 21 days) using an ALP Substrate Kit (Bio-Rad). Briefly, constructs (n=3 per group per time point) were homogenized in 500 μ l of 0.75 M of 2-amino-2-methyl-1-propanol (pH 10.3) on ice and stored at -20°C. Assay substrate solution was prepared according to the manufacturer's instructions. 120 μ l of sample solution was combined with 480 μ l of assay substrate solution and incubated at room temperature for 2 minutes; the absorbance of this solution was measured at 405 nm every 30 seconds for 7 minutes using a DU730 UV/Vis spectrophotometer (Beckman Coulter). A graph of absorbance vs. time was plotted and the slope was determined via a

linear fit to calculate the rate of reaction. A higher slope indicates increased ALP activity. ALP activity was expressed as change in absorbance per minute per construct wet weight.

4.7.12. Calcium content

Constructs were assayed for calcium content at 7 and 21 days. At each time point, cell-seeded as well as acellular cryogels (n=3 per group) were collected and lyophilized. After measuring their dry weight, lyophilized constructs were homogenized in 0.5 ml of 0.5 M HCl. The homogenates were vigorously vortexed for 16 hours at 4°C. The calcium concentration was measured spectrophotometrically at 570 nm via o-cresolphthalein complexone using a two reagent calcium kit (Pointe Scientific) according to the manufacturer's instructions. The total calcium content of each construct was normalized to its dry weight. Moreover, poly(ethylene glycol) hydrogels-containing A6ACA moieties have been previously shown to undergo mineralization in serum-supplemented solutions containing Ca^{2+} and PO_4^{3-} (Phadke *et al.*, 2010b). To correct for this, the Ca content of the corresponding acellular construct was subtracted from the average cellular Ca content of the cell-seeded constructs.

4.7.13. DNA content

Cell-seeded cryogels (n=3) were collected at 0d, 7d and 21d for analysis of DNA content. Samples were frozen at -80°C, lyophilized and digested in papain solution (0.125 mg/ml papain [Worthington Biochemicals], 10 mM phosphate and 10 mM EDTA, pH=6.3) for 16 h at 60°C. DNA was then measured in the papain digests using the Quant-IT Picogreen dsDNA kit (Invitrogen).

4.7.14. Mineralization of cryogels

Mineralized samples were prepared by a procedure described previously with some modifications (Phadke *et al.*, 2010b). Cryogels were immersed in DI H₂O for 24 hours, dried at 37°C and swollen in simulated body fluid (SBF) as detailed previously (Oyane *et al.*, 2003). The SBF-swollen cryogels were then partially dried for 60 minutes at room temperature on tissue paper to remove excess SBF from the pores and immersed in a solution of 40 mM Ca²⁺/24 mM HPO₄²⁻ at pH 5.2 for 1 hour on an orbital shaker at 300 rpm at 25°C. Following immersion, cryogels were rinsed briefly in running DI H₂O and incubated in SBF for 48 hours, with daily change of solution. The samples were then soaked in PBS for 6 hours, sterilized by immersion in 70% ethanol for 6 hours and incubated in sterile PBS for 3 days with at least 2X daily solution change to remove residual ethanol.

4.7.15. Effect of mineralization on cell viability

Columnar cryogels were mineralized 0, 1X and 2X respectively as detailed above. These were then soaked in hMSC growth medium for 18 hours, dried under sterile conditions for 105 minutes and seeded with hMSCs at passage 6 as described in the *in vitro* study. These were then cultured at 37°C, 5% CO₂ for 4 days in hMSC growth medium, cut into thin slices and stained using the Live-Dead staining (Invitrogen) as detailed above to assess cell viability.

4.7.16. Cell seeding of mineralized cryogels

For cellular mineralized constructs, 1X mineralized cryogels were incubated in hMSC growth medium for 18 hours, dried under sterile conditions for 105 minutes and

seeded with hMSCs at passage 6 as detailed above. Cell-seeded cryogels were incubated in growth medium at 37°C, 5% CO₂ for 1 week to allow for their attachment and proliferation prior to subcutaneous implantation

4.7.17. Subcutaneous implantation

Five athymic male rats, 12-months-old, weighing 400 to 450 g were used in the present study with the approval of the Institutional Animal Care and Use Committee. After intraperitoneal administration of ketamine hydrochloride (75 mg/kg, Ketaset®, Fort Dodge) and xylazine (3 mg/kg, AnaSed®, Akorn, Inc.), the rats were placed into a prone position and prepared in a standard surgical fashion. A single midline incision (1.5 mm) on the back of the rat was made, and four subcutaneous pouches (cranial-left and -right and caudal-left and -right) were constructed by blunt dissection using a 1 cm wide spatula. A cryogel (either mineralized- acellular, non-mineralized- acellular or mineralized- hMSC-seeded) was inserted into the end of each pouch. For acellular cryogels, one sample of each group (spongy mineralized, spongy non-mineralized, columnar mineralized, columnar non-mineralized) was implanted in each rat, with three rats receiving these acellular cryogels (four cryogels per rat). For the hMSC-seeded cryogels, two samples of each group were implanted in each of the remaining two rats (four cryogels per rat). The implantation sites for group were rotated in different rats to ensure that implantation site did not provide a bias. Following implantation, the skin was closed using staples.

4.7.18. Radiograph and sampling of implanted samples

Anterior-posterior (AP) digital radiographs (NAOMI; 356 dpi, 71 μm pixel size, Tokyo, Japan) of the rats were obtained (55kV, 10 μAs) pre- and post-operatively. Bone formation was evaluated radiographically at each time point (0-, 2-, 4-, 6- and 9-weeks after surgery). All animals were sacrificed with CO_2 at 9 weeks after surgery. Subcutaneously implanted samples were excised following animal sacrifice and photographed to assess host cell infiltration. Samples were then fixed in 4% PFA in PBS for 96 hours and stored in 70% ethanol at 4°C.

4.7.19. Histology for *in vitro* samples

Constructs cultured *in vitro* were collected at 21 days of culture for histology. Samples were fixed for 24 hours in 4% paraformaldehyde (PFA) and stored in 70% ethanol at 4°C. The fixed constructs were dehydrated in graded concentrations of ethanol followed by subsequent immersion in Histo-Clear (National Diagnostics), embedded in paraffin and cut into 10 μm thick sections.

Sections were then analyzed through immunofluorescent staining for osteocalcin. Briefly, the sections were deparaffinized in xylene and gradually rehydrated through a series of decreasing ethanol concentrations. The re-hydrated sections were blocked with blocking buffer consisting of 3% bovine serum albumin (Sigma), 0.1% Triton-X 100 (Sigma) in PBS for 30 minutes and then exposed to primary antibody (osteocalcin anti-mouse, monoclonal) (Abcam, ab13420) diluted 1:100 in blocking buffer for 1 hour. After washing sections with PBS (30 minutes), samples were exposed to secondary antibody (Alexa Fluor 568 goat anti-mouse) diluted 1:250 in blocking buffer for 60 minutes.

Following this, sections were washed with PBS for 30 minutes, mounted on glass slides with Vecta Shield/DAPI (Vector Laboratories) and visualized via fluorescent microscopy.

4.7.20. Micro-computed tomography of subcutaneously implanted samples

Samples were visualized through micro-computed tomography (micro-CT) simultaneously during fixation in PFA with the SkyScan 1076 High Resolution In-Vivo Micro-Computed Tomography Scanner (SkyScan, Belgium), at 9 μm /pixel resolution. Scans were reconstructed from projections using NRecon software (SkyScan, Belgium) and converted to 3-dimensional models using CTAn software (SkyScan, Belgium).

4.7.21. Histology of subcutaneously implanted samples

Samples were embedded in paraffin, cut into 4 μm - thick sections and stained either using hematoxylin-eosin (H & E) or through immunohistochemical staining. Paraffin embedding, sectioning and H & E staining was carried out by San Diego Pathologists Medical Group.

For immunohistochemical staining, unstained sections were deparaffanized in CitriSolv and gradually rehydrated through series of decreasing ethanol concentrations and then treated for 5 minutes with 0.05% SDS, 12.5 mM Tris, 96 mM glycine. Sections were then blocked with blocking buffer (3% bovine serum albumin, 0.1% Triton-X 100 in PBS) for 60 minutes at room temperature, following which they were exposed to primary antibody (osteocalcin anti-mouse, Abcam, ab14320) diluted 1:100 in blocking buffer for 18 hours at 4°C. Sections were then washed in PBS for 30 minutes and exposed to 0.3% hydrogen peroxide in PBS for 20 minutes at room temperature to block

endogenous peroxidase activity. Samples were exposed to HRP-conjugated secondary antibody (goat anti-mouse IgG-HRP, Santa Cruz Biotechnology, sc-2031) diluted 1:100 in blocking buffer for 60 minutes at room temperature, washed with PBS for 30 minutes and developed using a diaminobenzidine (DAB) substrate kit (Vector Labs) according to the manufacturer's instructions. Samples were then briefly washed with PBS to remove excess DAB and visualized using bright-field microscopy.

4.7.22. Histology of non-implanted mineralized cryogel

In order to account for the appositional mineralization of the mineralized scaffolds *in vivo*, a mineralized spongy cryogel was synthesized as detailed above and immersed in simulated body fluid (Oyane *et al.*, 2003) for 7 days at 37°C to simulate appositional, non-osteogenic mineralization of the scaffold under physiological conditions. This cryogel was then dehydrated, embedded in paraffin and cut into 4 µm-thick sections which were then stained with H & E and subsequently visualized with bright field microscopy.

4.7.23. Statistical analysis

The reproducibility of the *in vitro* effect of scaffold architecture on osteogenesis was confirmed through by repeating the experiments independently twice, beyond the replicates listed. Statistical significance was determined through two way analysis of variance (ANOVA), with Bonferroni post-test to compare individual groups ($p < 0.05$). Statistical analysis was carried out using Prism 4.6 (Graphpad)

4.8. Figures

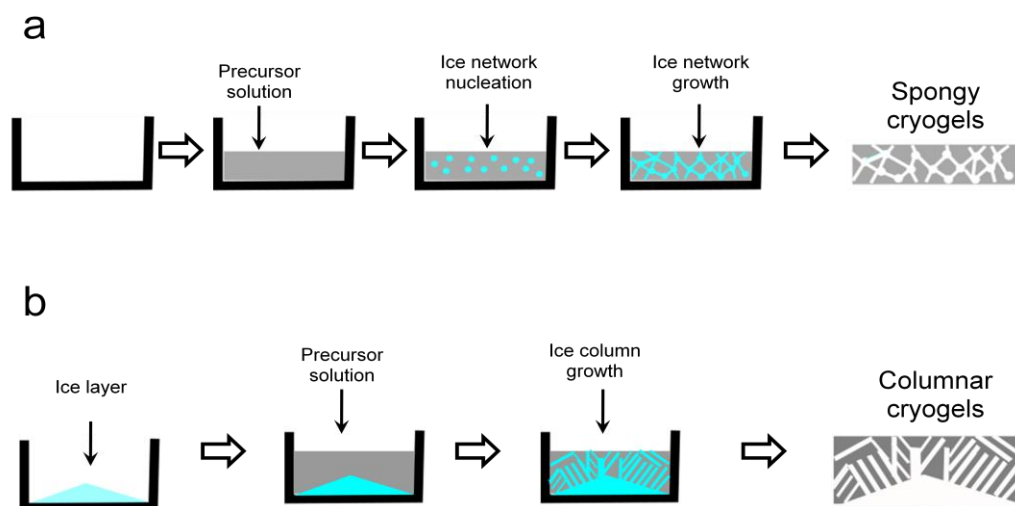


Figure 4.1. Synthesis of cryogel scaffolds

Schematic diagram detailing the synthesis of (a) Spongy and (b) Columnar cryogels

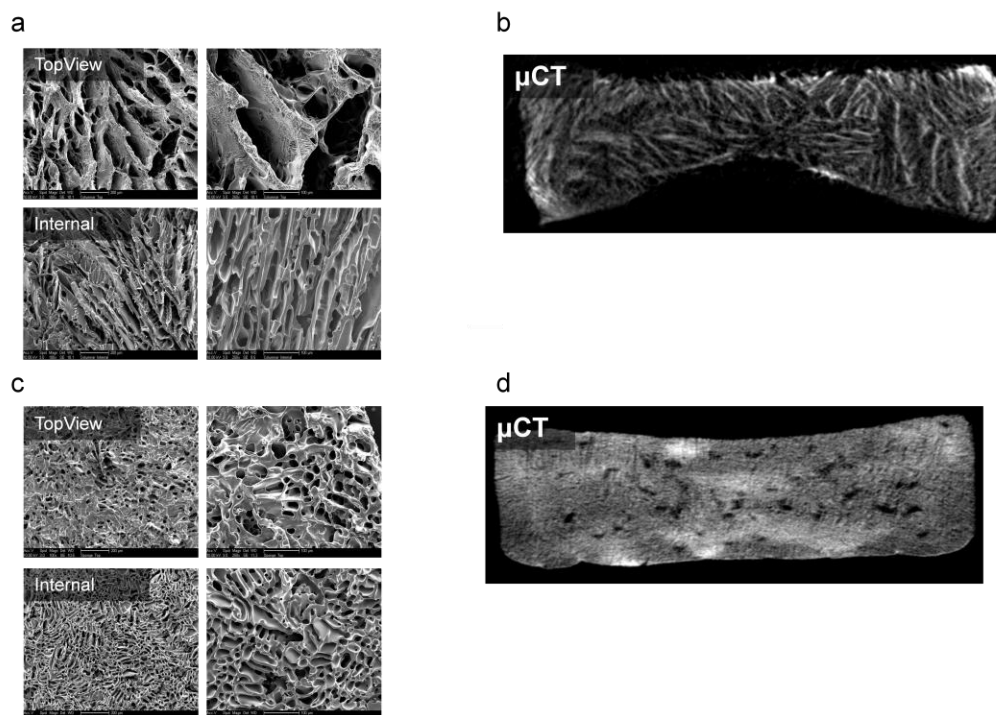


Figure 4.2. Morphology of spongy and columnar pore structure

(a-b) Columnar cryogels visualized through (a) scanning electron microscopy and (b) micro-computed tomography. (c,d) Spongy cryogels visualized through (c) scanning electron microscopy and (d) micro-computed tomography.

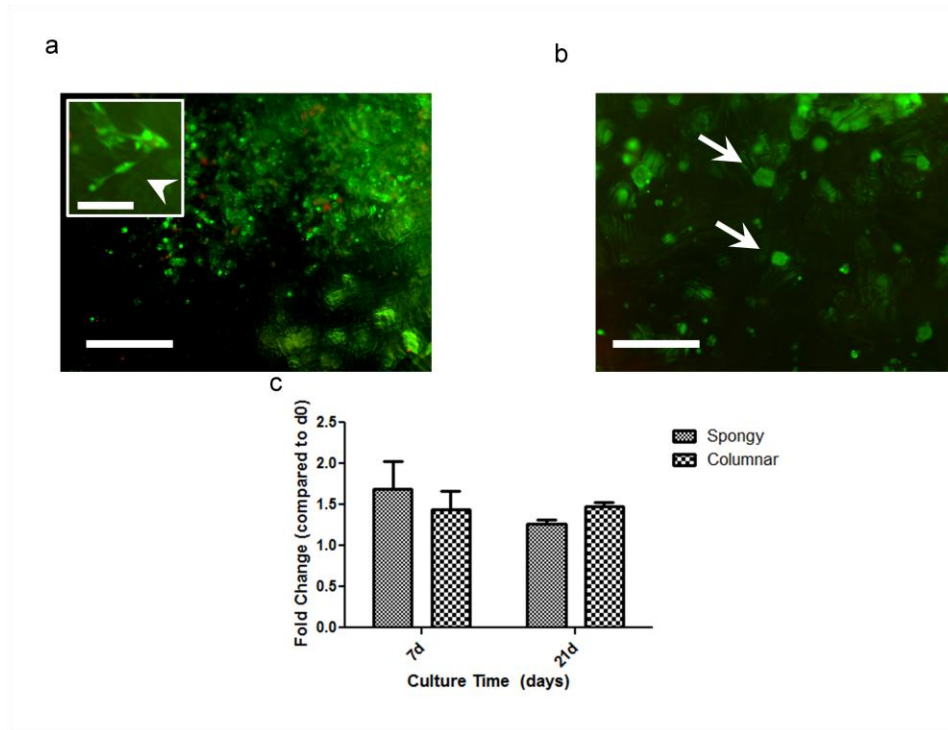


Figure 4.3. Cell attachment and proliferation to cryogels

Live-Dead staining of human mesenchymal stem cells (hMSCs) seeded in (a) spongy and (b) columnar cryogels. In (a), the arrowhead in the inset image indicates a spread cell inside a spongy cryogel. Scale bar: 80 μm . In (b), white arrows indicate cellular aggregates in a columnar cryogel. Live cells are stained green; dead cells are stained red. Scale bars in the main images represent 400 μm . (c) Proliferation of hMSCs in the spongy and columnar cryogels, calculated through DNA content and normalized to d0. Error bars represent standard error of the mean (n=3).

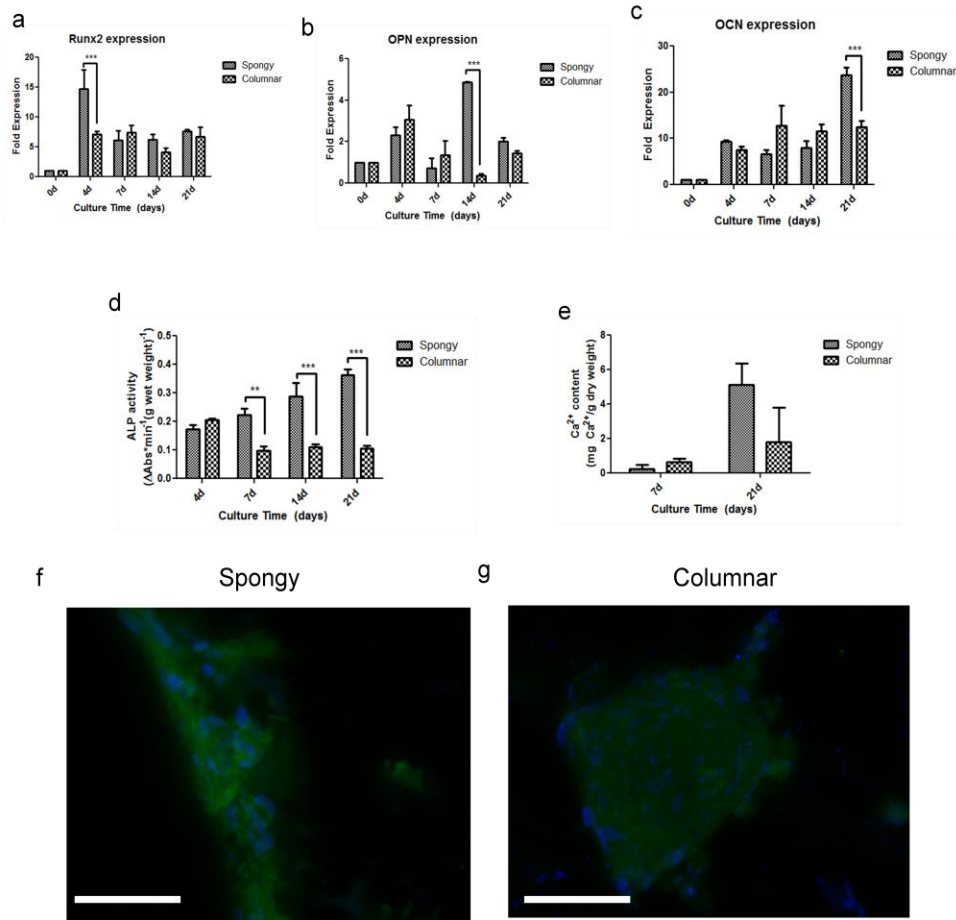


Figure 4.4. Osteogenic differentiation of hMSCs in cryogels *in vitro*

Expression of osteogenic markers (a) Runx2 (b) osteopontin (OPN) and (c) osteocalcin (OCN) in the spongy and columnar cryogels (d) Alkaline phosphatase activity of human mesenchymal stem cells (hMSCs) seeded on spongy and columnar cryogels (e) Calcium content of spongy and columnar cryogels at 7d and 21d of culture. Error bars represent standard error of the mean ($n=3$; **: $p<0.01$, ***: $p<0.001$). (f, g) Immunofluorescent staining for OCN at 21d in (f) Spongy and (g) Columnar cryogels. Green: OCN, Blue: Nuclei. Error bars represent $50 \mu\text{m}$

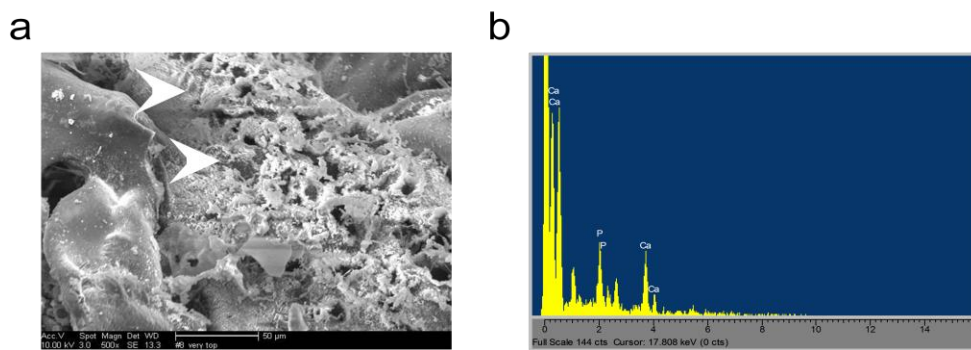


Figure 4.5. Mineralized cryogels

(a) Scanning electron microscopy image of mineralized A6ACA cryogel prior to implantation with (b) Elemental Dispersive Spectroscopy (EDS) spectrum, indicating presence of calcium and phosphate in the mineralized phase (indicated by white arrowheads).

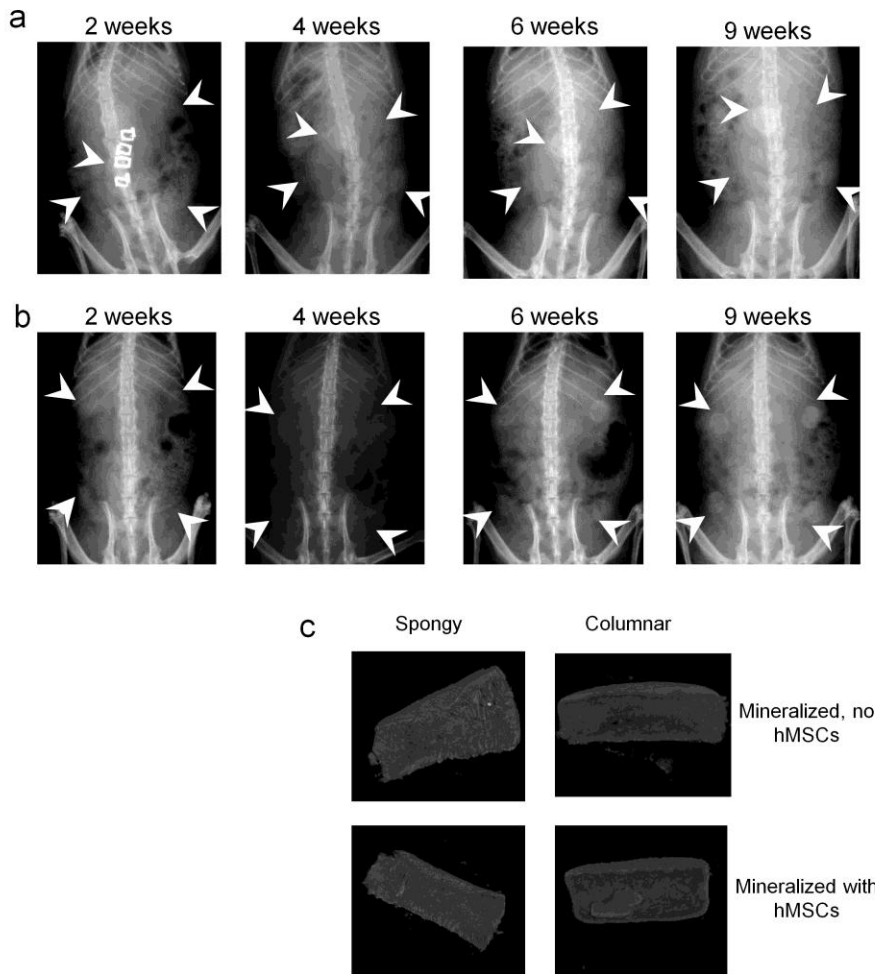


Figure 4.6. Ectopic bone formation evaluated radiographically.

(a, b) Digital X-rays for (a) acellular and (b) hMSC-seeded cryogels at various time points in representative animals. White arrowheads indicate the location of the implants. (c) 3D micro-CT models of the mineralized cryogels after 9 weeks of subcutaneous implantation. Non-mineralized hydrogels could not be imaged due to the lack of hard tissue formation.

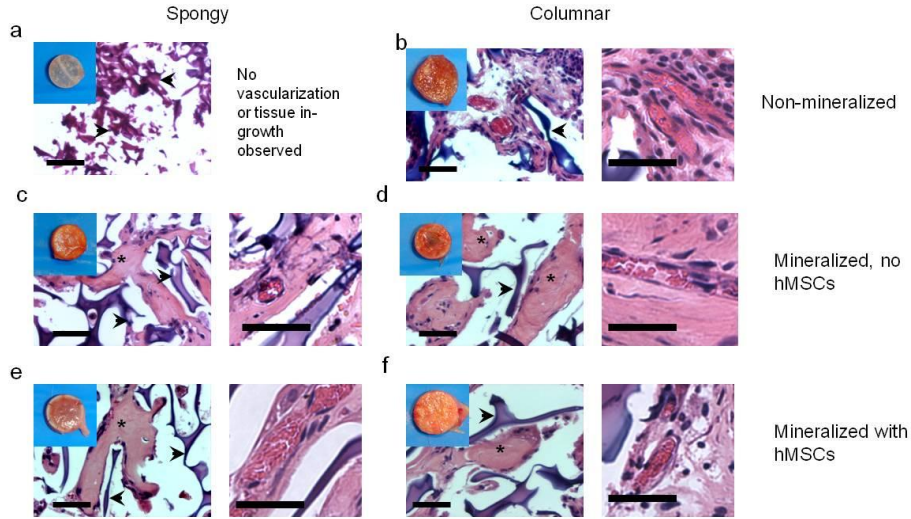


Figure 4.7. Histological analysis of bone formation and vascularization *in vivo*

H&E staining and gross photographs of constructs (inset) after 9 weeks of subcutaneous implantation. (a) non-mineralized, spongy cryogels (b) non-mineralized, columnar cryogels (c) mineralized, spongy cryogels without hMSCs (d) mineralized, columnar cryogels without hMSCs (e) mineralized, spongy cryogels with hMSCs (f) mineralized, columnar cryogels with hMSCs. Images on the right indicate the presence of lumen-like structures within the respective scaffold as evidence of vascularization. Arrowheads indicate the scaffold surface. All scale bars represent 50 μm .

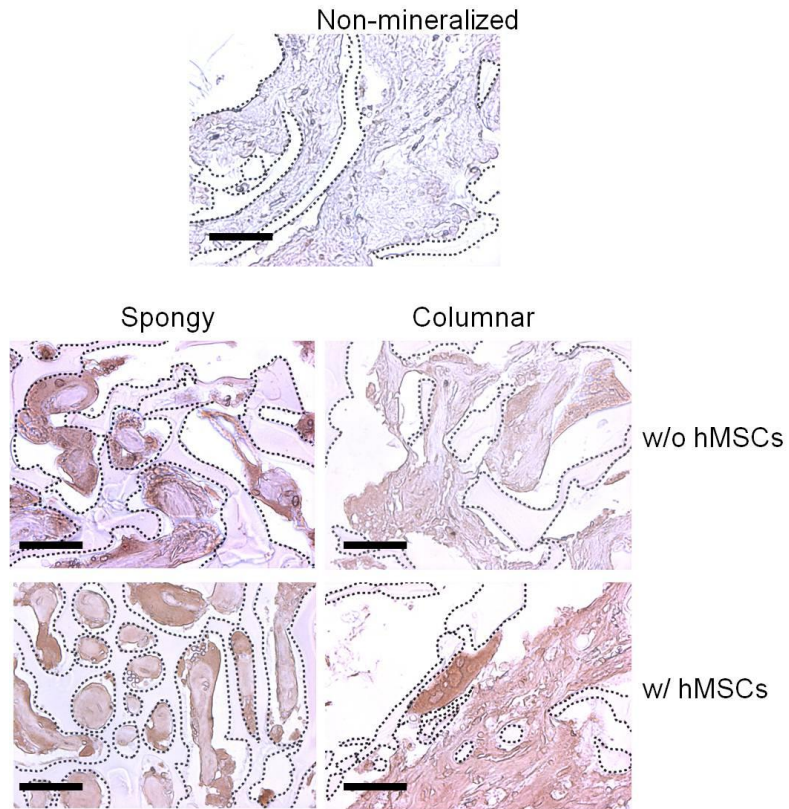


Figure 4.8. OCN expression in ectopic bone

Immunohistochemical staining for osteocalcin (brown) in cryogel constructs implanted subcutaneously in nude rats for 9 weeks. Dotted lines represent the scaffold surface. Scale bars represent 50 μm .

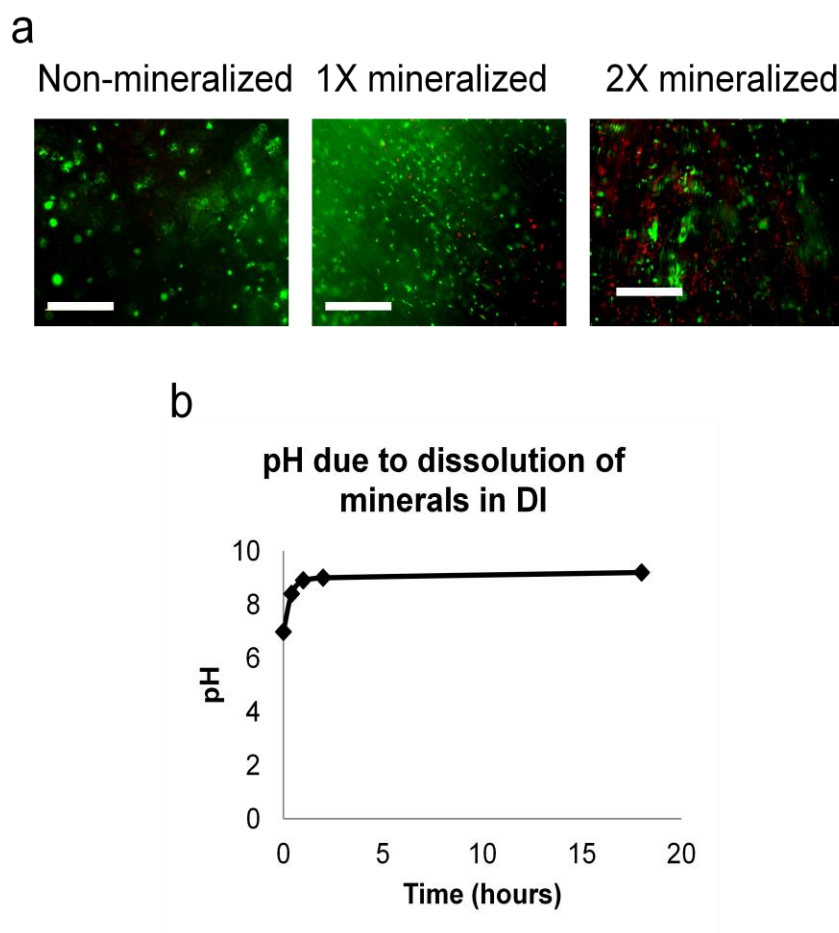


Figure 4.S1. Effect of amount of mineralization on cell viability

(a) Live-dead staining of hMSCs seeded on cryogels mineralized 0, 1 time and 2 times, respectively after 4 days of culture *in vitro*. Live cells are stained green, dead cells are stained red. Scale bars represent 500 μm . (b) pH of DI water after immersion of the 2X mineralized cryogels.

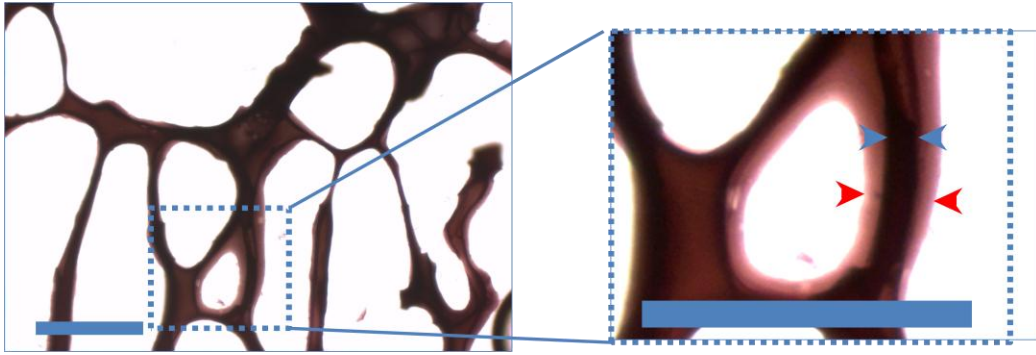


Figure 4.S2. Histology of non-implanted mineralized cryogels

H & E staining of mineralized cryogels allowed to undergo additional mineralization under physiological conditions *in vitro* to simulate appositional, non-osteogenic mineralization. Red arrows indicate the eosinophilic mineralized phase, blue arrows indicate the scaffold surface. Scale bars represent 100 μm .

4.9. Tables

Table 4.1. Microstructural parameters of scaffolds

Properties	Unit	Spongy	Columnar
Total Pore Area	m ² /g	0.37±0.18	0.17±0.05
Median Pore Diameter (Volume)	μm	28.27±11.99	60.07±16.36
Median Pore Diameter (Area)	μm	19.27±6.88	30.37±7.75
Average Pore Diameter (4V/A)	μm	26.69±10.59	45.56±9.93
Porosity	%	70.0± 0.4	70±5

Table 4.2. List of primers used for real-time PCR.

Gene	F/R	Primer Sequence	Amplicon size (bp)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F	5' CAT CAA GAA GGT GGT GAA GC 3'	177
	R	5' GTT GTC ATA CCA GGA AAT GAG C 3'	
Osteocalcin (OCN)	F	5' GAA GCC CAG CGG TGC A 3'	70
	R	5' CAC TAC CTC GCT GCC CTC C 3'	
Runt-related Transcription Factor 2 (Runx2)	F	5' CCA CCC GGC CGA ACT GGT CC 3'	258
	R	5' CCT CGT CCG CTC CGG CCC ACA 3'	
Osteopontin (OPN)	F	5' CCA AGT AAG TCC AAC GAA AG 3'	348
	R	5' GGT GAT GTC CTC GTC TGT A 3'	

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CHAPTER 5: Synthetic biomineralized matrices as osteoinductive bone grafts for posterolateral lumbar fusion: an *in vivo* study

5.1. Abstract

Spinal fusion procedures are becoming increasingly prevalent and often require the use of bone grafts. Due to the issues associated with autografts, allografts, xenografts and recombinant growth factors, there is a pressing need for the development of synthetic materials that can serve as effective bone grafting substitutes. This study involved evaluation of synthetic grafts consisting of biomineralized porous matrices of N-acryloyl 6-aminocaproic acid-co-poly(ethylene glycol) diacrylate (A6ACA-PEGDA) in a rodent posterolateral fusion model. Fourteen athymic male rats underwent a posterolateral lumbar fusion procedure, with seven rats receiving grafts soaked in bone marrow flush from syngeneic donors, and seven rats receiving grafts soaked in physiological saline. Both bone marrow flush-soaked and saline-soaked grafts showed rapid bone formation, with the formation of well-defined fusion masses at 4 weeks in all animals. Radiographic and mechanical lateral bending analyses revealed that both marrow flush-soaked and saline-soaked grafts promoted spinal fusion compared to sham surgery to a similar extent. These synthetic biomineralized matrices thus hold great promises as osteoinductive matrices for spinal fusion procedures, without requiring the addition of exogenous cells or biologics.

5.2. Introduction

Bone grafting has become an increasingly prevalent procedure, with over 500,000 bone grafting procedures performed annually in the United States alone (Faour *et al.*, 2011); bone is the third most transplanted tissue after skin and blood. A variety of procedures necessitate the use of bone grafts: these include spinal fusion procedures and large bone defects arising from trauma or excision of large segments of bone due to infection or tumor invasion. Among these, spinal fusions are increasingly prevalent; approximately half of the bone grafting procedures performed annually are related to spinal fusion. Spinal fusion procedures are typically used to treat conditions arising from damage to the intervertebral disc, nerve compression and spondylolistheses among others.

Damage to intervertebral discs is often treated by interbody fusion, wherein the damaged disc is removed and the graft is placed between the vertebral bodies, leading to their fusion. Spondylolisthesis on the other hand, is often treated via posterolateral fusion. Posterolateral fusion involves the fusion of adjacent vertebrae by placement and securing of the bone graft between the transverse processes of adjacent vertebrae following their decortication. This leads to newly formed bone attaching to the transverse processes, resulting in the fusion of the vertebrae and preventing their independent motion. This is considered a fairly challenging model for bone graft evaluation, and for a variety of reasons. First and foremost, this procedure necessitates the formation of new bone between transverse processes and thereby requires that the grafting material possess osteoinductivity, in addition to osteoconductivity. Secondly, despite being among the most prevalent of spinal fusion procedures performed, posterolateral intertransverse

lumbar fusions are susceptible a high rate of non-unions i.e. the failure to form a solid mass of bone, with non-union rates varying between 10 and 40% for single-level fusions in previous studies (Boden, 2000). This suggests that there is currently an insufficient understanding of the physiological and cellular processes that contribute towards successful bone formation and subsequent fusion in these procedures, further underscoring the highly challenging nature of this procedure both, in human patients as well as in animal models (Boden *et al.*, 1999). A factor that makes this procedure particularly challenging in small animal models such as rats and rabbits is the destabilization of the grafts due to the lack of internal fixation on account of the small size of the animal spines, thereby increasing the risk of non-union.

Current grafting strategies include the use of autografts, allografts, xenografts and synthetic substitutes (Vaz *et al.*, 2010). Autografting involves using bone from the same patient (or from syngeneic patients in the case of rodent models), typically extracted from the iliac crest. This has traditionally been considered a gold standard, although in the literature, success rates in spinal fusion procedures with autograft have varied from as low as 30% to 100% in reported studies (Grauer *et al.*, 2004). Despite their reported excellent clinical performance, autografts suffer from a number of issues, the most notable being donor site morbidity. The grafting procedure is highly invasive and often results in extended recovery time, and can often result in chronic pain. Additionally poor bone repair capacity in specific patient populations such as the elderly, patients suffering from osteoporosis and heavy smokers can preclude the use of autografting. Allografts are typically sourced from cadaveric bone and have been used to mitigate some of the

aforementioned issues facing autografts. However, allografts suffer from the risks of immunorejection and disease transmission, require careful handling and storage to preserve their viability and also are subject to significant batch-to-batch variability, making them less than ideal as substitutes for autologous bone grafts.

An analog to allografts that has been extensively evaluated on account of its osteoinductive potential is demineralized bone matrix (DBM). Urist and colleagues demonstrated that demineralization of bone dramatically increased its bone induction capacity (Urist, 1965; Urist *et al.*, 1984); subsequent work showed that this was likely due to enhanced exposure of the host tissue to the osteoinductive bone morphogenetic proteins present within the DBM, as evidenced by the enhanced osteoinductive potential of specific protein fractions extracted from the DBM (Urist, 1965; Urist *et al.*, 1984). In more recent times, this finding has led to the extensive use of synthetic implants utilizing recombinant bone morphogenetic proteins (BMPs), with rhBMP-2 and rhBMP-7 currently approved for clinical use (Bhatia and Bhatia, 2012; Glassman *et al.*, 2011). Subsequent studies have demonstrated that rhBMPs are powerful inducers of bone formation and in spinal fusion procedures have produced clinical outcomes superior to even those obtained through autografts (Burkus *et al.*, 2003; Dimar *et al.*, 2006). A number of studies have noted that BMP-based graft substitutes have provided favorable clinical outcomes even in patient subpopulations such as the elderly (Glassman *et al.*, 2008) and heavy smokers (Glassman *et al.*, 2007), all of which are typically associated with poor fusion outcomes even through autografting. Despite these obvious advantages, BMP-based strategies suffer from a number of shortcomings. First and foremost, the low

half-life of BMPs in physiological conditions necessitates the delivery of a very large dose, typically at least three orders of magnitude higher than typical concentrations observed *in vivo*. Besides the high costs associated with this dosage, recent studies have also noted that the use of BMPs for spinal fusion can produce serious side effects such as respiratory effects, ectopic bone formation, local bone resorption at the implant site and even an increase in the risk for cancer (Carragee and Weiner, 2011).

Due to the aforementioned issues with the use of autografts, allografts and recombinant growth factors, recent years have witnessed an emphasis on the development of completely synthetic material-based grafts. These typically mimic the mineralized phase of bone and are composed of crystalline calcium phosphate, silica-containing bioactive glasses and may contain trace amounts of elements such as Sr^{2+} to enhance the osteogenic response (Barrère *et al.*, 2006). These materials are relatively cheap to produce, completely defined and typically highly biocompatible, underscoring their practicality as use in bone grafting. A number of studies have demonstrated the ability of these materials to at least support osteogenic differentiation of progenitor cells, and in some cases, even promote the osteogenic differentiation of progenitor cells in the absence of exogenously added soluble osteogenic stimuli. *In vivo*, these materials have demonstrated ectopic osteoinduction in large animal models such as dogs, pigs, sheep and primates, and have also demonstrated applicability in spinal fusion models in these species (Yuan *et al.*, 2010). However, these materials have rarely demonstrated osteoinductivity during spinal fusion procedures in the absence of exogenously added cells or biologic factors especially in rodent models. A synthetic graft capable of inducing

bone formation and spinal fusion without the addition of biologics such as bone marrow aspirate would be highly desirable especially due to the invasive nature of the marrow harvesting procedure.

In a previous study, we have demonstrated that biomineralized matrices of N-acryloyl 6-aminocaproic acid-co-poly(ethylene glycol) diacrylate (A6ACA-PEGDA) have the ability to promote osteogenesis of human mesenchymal cells in vitro without the use of soluble osteogenic stimuli (Phadke *et al.*, 2012c). These materials have also been shown to promote ectopic bone formation when subcutaneously implanted in rats (Phadke *et al.*, 2012a); thus, these materials have thus been demonstrated to exhibit osteoinductive potential without the use of exogenously added cells or osteoinductive recombinant growth factors in rats, considered particularly challenging for material-induced osteoinduction. These findings suggest that the biomineralized A6ACA-PEGDA matrices possess osteoinductivity and are highly promising as synthetic bone grafts. In this work, we have examined their ability to promote spinal fusion in nude rats

5.3. Results

5.3.1. Scaffold characterization

Our previous work suggested that macroporous scaffolds with spherical pores were more conducive to osteogenesis than scaffolds with a lamellar pore structure (Phadke *et al.*, 2012a). However, the cryogelation approach utilized previously yielded a relatively small pore size ($\sim 50 \mu\text{m}$); in order to increase the pore size while maintaining similar pore morphology, we decided to use a previously demonstrated approach utilizing

a microsphere-based template. Figure 5.1. is a schematic representation of the experiments.

Non-mineralized grafts showed a series of well-interconnected, spherical pores measuring approximately 100-150 μm in the dried state, and no evidence of calcium phosphate deposition confirmed by EDS. Scanning electron microscopy of the mineralized grafts revealed a similar pore morphology and size as the non-mineralized grafts, but with a surface layer of calcium phosphate coating the inner pore surface. EDS confirmed the calcium phosphate composition of this layer, with a Ca/P ratio of approximately 1.3 (Figure 5.2).

5.3.2. Radiographic evaluation and Micro-CT

No adverse reaction to the grafts was observed in any of the animals throughout the study period. Radiographic analysis of the animals immediately post-implantation revealed that the scaffolds were not visible in any of the animals. In contrast to the post-implantation radiographs, fusion masses of newly formed bone were visible in some of the animals as early as 2 weeks (3 out of 7 or 43% in saline, 4 out of 7 or 57% in marrow). By 4 weeks, all animals showed clear evidence of newly formed bone, with progressively increasing bone formation observed in radiographs at 6 and 8 weeks respectively (Figure 5.3.). Importantly, new bone formation was observed irrespective of whether the animals received grafts soaked in physiological saline, or bone marrow flush, and no observable difference could be discerned between animals from the two previous groups. Radiographic evaluation of fusion at 4, 6 and 8 weeks suggested a 100% fusion rate, as defined by the presence of newly formed bone bridging the transverse processes

at the fusion level. As the scaffolds were not visible at day 0, it was clear that the appearance of the fusion masses was due to new bone formation and not an artifact due to visualization of the grafts themselves. In contrast, previous work as well as other studies have revealed no bony fusion mass formation in animals subjected to sham surgeries (with no graft placement) (Yamaguchi *et al.*, 2012).

Micro-CT at 4 weeks revealed a similar amount of bone present in the fusion masses relative to total tissue volume, for both groups (approximately 30%). Visual inspection of micro-CT scans indicated that the formation of new bone was restricted to the grafts (Figure 5.4b-e.), while micro-CT at 0 weeks revealed that the grafts themselves were not radio-opaque or visible in micro-CT presumably due to the similar density between the soaked grafts and surrounding soft tissue. At 8 weeks, the bony fusion masses persisted and increased in density. Additionally, examination of transaxial views in the reconstructed 3D models revealed that the newly formed bone in the fusion mass attached with the decorticated transverse processes (Figure 5.4e). Further examination in the fusion masses revealed that bone formation was typically formed in the form of an exterior shell for larger graft fragments; smaller fragments on the other hand, showed excellent in-growth of bone. Additionally, while the graft fragments showed excellent bone formation, spaces between the fragments showed more sparse bone formation and presumably filled with soft tissue.

5.3.3. Manual palpation

Manual palpation revealed an average score of 3.0 for the marrow-soaked group and 2.9 for the saline soaked group, thus providing evidence of fusion in both groups,

with no significant differences between fusion indices. In comparison, we demonstrated in a previous study that by this scale, sham groups consistently scored approximately 1, supporting the radiographic observations of lack of fusion/bone formation in that group (Yamaguchi *et al.*, 2012).

5.3.4. Lateral bending analysis

A commonly stated shortcoming of manual palpation is its subjectivity. While previous studies have indicated that manual palpation of the spine is currently the gold standard for evaluation of spinal fusion in human patients (Boden *et al.*, 1995), the small size of the vertebrae in rats necessitate the use of a more objective mechanical test to validate the findings from manual palpation. As a result, we utilized a recently described technique involving the characterization of lateral bending of the fused spines upon controlled displacement (Yamaguchi *et al.*, 2012) (Figure 5.5.). Spines implanted with both, marrow-soaked and saline-soaked grafts showed a statistically significant reduction ($p < 0.05$) in lateral bending compared to the sham surgery group, with a lateral bending angle of approximately 50% of that observed in the sham surgery group. This reduction in mobility suggested that the grafts were able to efficiently promote fusion of the transverse processes through the formation of bridging bone. Furthermore, no difference was seen between the marrow-soaked or the saline-soaked group, further supporting the conclusions from the radiographic and micro-CT analyses that the saline-soaked grafts were able to promote fusion to a similar extent as the marrow flush-soaked grafts.

5.3.5. Histological evaluation

Examination of H & E staining for the decalcified sections from within the fusion masses revealed a combination of soft fibrous tissue and bone tissue. The bone tissue was observed to be lining the pores, appositional to the scaffold (Figure 5.6.). Some of the pores were also filled with bone –like tissue, supporting the findings of osteoinduction as detailed by micro-CT. Bone was observed to be present in the pores both, in the region appositional to the decorticated transverse processes as well as in the interior of the fusion mass.

5.4. Discussion

Our study describes the evaluation of synthetic composite grafts in a rodent posterolateral fusion model. Recent work has probed synthetic calcium phosphate-based materials as potential alternatives to autografting and allografting, as well as current recombinant factor-based strategies (Yuan *et al.*, 2010). Among these, β -TCP and biphasic calcium phosphates (containing a combination of β -TCP and hydroxyapatite) have yielded particularly promising results. Studies have also demonstrated that doping the calcium phosphate matrix with components such as Sr^{2+} ions and silica can dramatically improve the osteogenic response of these materials (Barrère *et al.*, 2006; Jenis and Banco, 2010). While such materials have conclusively demonstrated reproducible osteoinduction upon ectopic implantation (either at subcutaneous or intramuscular sites) in large animal models such as goats, dogs, sheep, pigs and primates, material-induced osteoinduction is relatively rare in rodent models, despite the potent induction of bone formation by cells and biologics in these animals (Yuan *et al.*, 2006).

Previous studies that have utilized synthetic matrices for posterolateral fusion in small animal models have typically utilized biologic additives such as exogenously added cells (Kai *et al.*, 2003), demineralized bone (Lee *et al.*, 2005), bone marrow aspirate (Tay *et al.*, 1998), or platelet-rich plasma (Okamoto *et al.*, 2012) and in fact have reported significantly worse fusion outcomes in the absence of these additives. In one such recent study, β -TCP/gelatin composite sponges demonstrated the ability to promote posterolateral fusion in rats when administered in combination with platelet-rich plasma (Okamoto *et al.*, 2012); in that study, the composite sponges without PRP were able to induce only a modest improvement over sham surgery, but were significantly worse than the PRP-soaked sponges. Another recent study found that composites of type I collagen and hydroxyapatite were able to promote fusion in a rabbit posterolateral fusion model both with and without bone marrow aspirate (Walsh *et al.*, 2009). In that study however, histology revealed that the composites required the addition of bone marrow aspirate for the formation of bone away from the transverse processes, in the center of the fusion mass. Yet another study investigated the effect of adipose derived stem cells on posterolateral fusion in Fisher rats utilizing a commercially available β -TCP matrix and found similar fusion scores both with and without the addition of stem cells, although histological analysis in that study revealed significantly higher lamellar bone formation in the stem cell-seeded blocks (Lopez *et al.*, 2008).

It is thus evident that in small animal models, currently-utilized calcium phosphates lack the fusion efficacy displayed in large animal models. Osteoinduction in rodents could thus be an indicator for excellent osteoinductivity in other animals and

humans as well, leading to our choice of a rat model. We chose nude rats specifically as our previous study showed that biomineralized A6ACA matrices can promote osteoinduction in this strain; as recent studies have demonstrated that osteoinductive capacity of synthetic materials can vary greatly across different rodent strains (Barradas *et al.*, 2012), we decided to keep the strain consistent with our previous study.

A potential confounding factor in radiographic evaluation of fusion can be artifacts arising from the presence of a radio-opaque graft, which can be difficult to distinguish from neo-bone tissue. In our case however, X-ray images and micro-CT analysis of the grafts immediately following implantation showed that the grafts themselves were not visible as hard tissue; this is likely due to the fact that the grafts contained only a thin layer of calcium phosphate (measuring a ~5-10 microns thick). The subsequent fusion masses of hard tissue formed at the implantation site can thus be solely attributed to the formation of hard neo-bone tissue. The formation of fusion masses even in the saline-soaked grafts between 2-4 weeks and their progressive increase in radio-opacity suggests that the implanted grafts recruited host progenitor cells and then subsequently directed their differentiation into the osteoblastic lineage, leading to the formation of neo-bone tissue. It is important to note that during this study, several of the large graft fragments implanted showed neo-bone formation predominantly as a shell-like surface layer, although smaller fragments showed excellent in-growth of the newly formed bone tissue. The formation of an exterior shell has often been reported during bone induction in soft tissues, particularly when calcium phosphate matrices are used either in isolation or as carriers for osteoinductive growth factors (Hartman *et al.*, 2005;

Matsushita *et al.*, 2004); this could be due to the fact that the matrices possess osteoconductivity but may lack sufficient osteoinductivity to promote bone formation in their interior. In contrast, fusion masses in our study showed bone formation even in graft fragments located in the center of the fusion mass, away from the transverse processes thus indicating that the matrices were osteoinductive and not merely osteoconductive.

In addition to radiographic and histological evaluation, it is extremely important to evaluate the success of fusion mechanically. Manual palpation of the spine has commonly been used to evaluate fusion (Yee *et al.*, 2004). This involves observers comparing the range of motion observed at the fused levels to that observed in the non-fused adjacent levels and then grading the level of fusion. While this method may seem fairly subjective and susceptible to inter-observer and intra-observer variability, previous studies have shown that analysis via manual palpation does indeed show correlation with other methods of evaluation of fusion, although manual palpation tends to yield lower results than imaging-based methods (Yee *et al.*, 2004). The results of manual palpation in our study indicate that the grafts were able to promote fusion (i.e. a significant reduction in range of motion) both with and without addition of bone marrow. In addition, the lateral bending analysis provides a more objective measure of fusion and indicated that both, saline- and marrow flush-soaked synthetic biomineralized matrices led to a 50% reduction in 3D lateral bending angle when compared to the corresponding value from sham surgery in our previous work. Mechanical analysis thus corroborated the radiographic findings of fusion promoted by the synthetic grafts.

It is a particularly intriguing observation that the saline-soaked grafts performed similar to the grafts soaked in bone marrow flush. Our findings agree with our previous work, wherein biomineralized porous A6ACA-PEGDA scaffolds were able to induce ectopic bone formation upon subcutaneous implantation in nude rats, irrespective of whether the scaffolds were pre-seeded and cultured with human mesenchymal stem cells prior to their implantation (Phadke *et al.*, 2012a). In the previous study, the presence of the mineralized phase also dramatically improved the vascularization of the scaffolds. This indicates that the mineralized composite scaffolds have the ability to recruit host progenitor cells and stimulate their differentiation into an osteogenic lineage, leading to the deposition of bone matrix. We believe that there are a number of reasons underlying the osteoinductivity of our material. First and foremost, the biomineralization process wherein the organic template mimics the mineralization process observed in native bone and thus could potentially possess many of the physicochemical cues present in native mineralized bone tissue. It is possible that the semicrystalline nature of the biomineralized phase in the mineralized A6ACA grafts allowed for an increased level of dynamic dissolution-reprecipitation, as compared to the highly crystalline calcium phosphate based materials typically investigated for bone regeneration. *In vitro* studies have illustrated this dissolution-precipitation as playing a critical role in the osteoinductivity of calcium phosphate based materials (Chai *et al.*, 2010; Chai *et al.*, 2011; Phadke *et al.*, 2012b; Phadke *et al.*, 2012c). The mineralized phase could also promote the selective adsorption and subsequent release of pro-angiogenic and pro-osteogenic growth factors from the surrounding physiological environment (Lee *et al.*, 2011), leading to their concentration within the implant and fusion bed.

In human patients, biphasic calcium phosphates have shown the ability to promote posterolateral fusion when combined with morcelized autologous bone, yielding performances comparable to that obtained when using autograft alone (Dai and Jiang, 2008; Fujibayashi *et al.*, 2001); recently, silicated calcium phosphates have demonstrated the ability to promote fusion in combination with bone marrow aspirate (Jenis and Banco, 2010). However, current reports suggest that in the absence of biologic additives such as bone marrow, cells or demineralized bone matrix, synthetic calcium phosphates do not promote posterolateral fusion in human patients (Acharya *et al.*, 2008). These reports thus indicate that these synthetic materials do not possess many of the risks associated with recombinant factor-based therapies, such as osteolysis and ectopic bone formation, although currently-used synthetics typically require the addition of biologic material such as bone marrow aspirate. Given the relative scarcity of reports of biologic-independent bone induction in both, human patients and rodent models by calcium phosphates, the consistent osteoinductivity displayed by the mineralized A6ACA grafts in our study is highly encouraging and could potentially allow for their use in spinal fusion procedures in patients without the need for extraction of bone marrow aspirate, platelet-rich plasma or other autologous biologic materials. Synthetic materials such as these biomineralized grafts that could promote fusion without the addition of biologics would thus be a highly desirable alternative to current grafting strategies.

5.5. Conclusions

This work demonstrates the utility of novel synthetic bone grafts based on biomineralized porous matrices in an athymic rat posterolateral fusion model. These

synthetic grafts demonstrated osteoinduction and were able to promote posterolateral fusion as evidenced by radiographic, histological and lateral bending analyses. These grafts were able to promote fusion irrespective of the presence of syngeneic bone marrow flush, as both saline-soaked and bone marrow flush-soaked grafts were able to produce highly similar, statistically significant improvements when compared to sham surgery.

5.6. Experimental Section

5.6.1. Graft preparation

Porous PEGDA-A6ACA hydrogels were prepared using a template-leaching method similar to that previously described (Bryant *et al.*, 2007). Briefly, 110 mg of PMMA microspheres (mean diameter: 165 μm) (Bangs Laboratories, MI) were poured into cylindrical polypropylene molds measuring 7 mm in diameter and tapped vigorously for 20-30 seconds to ensure optimal packing of microspheres. The microsphere-filled molds were sintered at 140°C for 26 hours and allowed to cool to room temperature for at least 4 hours. A precursor solution was prepared by adding 200 mg/ml of poly(ethylene glycol)-diacrylate (Mn: 3.4 kDa) to a 0.5M solution of N-acryloyl 6-aminocaproic acid (A6ACA) in 0.5M NaOH and chilled on ice for 15 minutes. Polymerization was initiated by the addition of 0.5% ammonium persulfate and 0.07% N,N,N',N'-tetramethylethylenediamine; 0.110 ml of the solution was immediately poured into each mold and allowed to vacuum-infiltrate by cycling the pressure between ambient pressure and 28 In.Hg vacuum every 5 minutes, with 3 cycles. The infiltrated samples were then allowed to polymerize under humid conditions at 37°C for 2 hours, forming composites of the PMMA template-embedded hydrogels. PMMA templates were removed by

washing in acetone for 3 days (with 2X daily change of solution), and then subsequently by immersing the hydrogels in decreasing concentrations of acetone (90%, 70%, 30%, 0%) for 24, 12, 6 and 6 hours respectively. The porous hydrogels were also subsequently washed in DI water for at least 3 days (with 3X daily change of solution) to remove traces of acetone, then scraped to remove any thin non-porous layer at the surface and dried at 37°C for 24 hours.

Grafts were mineralized using a previously described procedure with some modification (Phadke *et al.*, 2012a; Phadke *et al.*, 2012c; Phadke *et al.*, 2010). Briefly, they were re-swollen in simulated body fluid (m-SBF), prepared as detailed previously (Oyane *et al.*, 2003) for 12 hours, minced into pieces with a surgical scalpel and then mineralized by immersion in 40 mM Ca^{2+} /24 mM PO_4^{3-} (pH raised from storage pH of 2.0 to 5.2 with 1 M Tris-HCl, pH 7.5 just prior to usage) under vacuum cycling to ensure optimal infiltration of mineralization solution into scaffold interior for 1 hour. Excess mineralization solution was then removed from the graft pores, and the grafts were immersed in fresh m-SBF for an additional 48 hours. Grafts were then washed briefly in sterile PBS for 6 hours to remove excess m-SBF from the pores, loaded into 1 ml syringes (Terumo Medical Inc) (0.3 cc/syringe), frozen at -80°C and lyophilized for 48 hours.

5.6.2. Surgical procedure

Sixteen male athymic rats (*rnu/rnu*, Harlan) weighing 250-300 g were used for the study with the approval of the Institutional Animal Care and Use Committee. An additional two animals were used as bone marrow donors and sacrificed with CO_2 . The

femurs were removed and cleaned of soft tissue remnants. The ends of the femur were cut and the femurs were flushed 4X with DMEM (GIBCO) (4 ml/femur total). The concentration of viable mononucleated cells was determined to be 1.1×10^7 cells/ml. The remaining 14 animals were used for implantation. Animals were anesthetized with an intramuscular injection 0.45 ml of ketamine-xylazine. L4-L5 transverse processes were exposed through two dorsal midline incisions under general anesthesia, stripped of periosteum by scraping and decorticated with a burr until bleeding was observed from the transverse processes. 0.3 cc of the synthetic graft was rehydrated in either physiological saline or bone marrow flush and placed between the transverse processes in the fusion bed on each side of the spine. Half of the animals (n=7) received bone marrow flush-soaked grafts, while the other half (n=7) received grafts soaked in physiological saline. The muscle pouch was closed with resorbable Vicryl sutures and the skin was closed with staples. Buprenex was administered for post-operative pain and the animals were allowed access to food and water *ad libitum*.

Following 8 weeks, rats were euthanized by CO₂ and cervical dislocation.

5.6.3. Radiographic Monitoring

Bone formation in the live rats was monitored biweekly through anterior-posterior (AP) digital radiographs (NAOMI; 365 dpi; 71 μ m pixel size; 50 kVp; 10 μ As) at 0, 2, 4, 6 and 8 weeks. Post-sacrifice, animals were also imaged via high resolution X-ray films using a tabletop Faxitron X-ray machine.

5.6.4. Micro-computed tomography

Animals were imaged via whole-body live micro-computed tomography (Skyscan 1076, Skyscan, Belgium) at 4 and 8 weeks (pixel size: 35 μm ; 100 kV; 1.0 mm Al filter) under anesthesia (2% isoflurane). Scans were reconstructed using NRecon software and analyzed via CTAn and DataViewer software (Skyscan, Belgium).

Bone volume percentage was calculated for each animal at 8 weeks from the reconstructed micro-CT scans using CTAn software. Briefly, a thresholding value was determined by visual inspection of native trabecular bone that background could be minimized while minimizing threshold-mediated loss of trabecular bone from the image. The fusion mass region on each side of the spine (left and right) was then isolated as a region of interest in each slice via visual inspection and the determined threshold was applied. Bone volume was quantified as percentage of volume occupied by bone (hard tissue with a pixel value exceeding the threshold) as compared to total tissue volume for each side. For each animal, bone volume percentage was expressed as the average of the bone volume percentage in the left and right side fusion mass respectively.

5.6.5. Manual Palpation

Following sacrifice, spines were extracted from animals and manually palpated to evaluate fusion. Movement of vertebra at fused levels were compared to movement of vertebra at adjacent, unfused levels (Boden *et al.*, 1995). Spines were graded on a scale of 1-4, with 1 representing no fusion with substantial movement, 2 representing limited fusion with reduced movement, 3 representing substantial fusion with minimal movement and 4 representing complete fusion with no detectable movement.

5.6.6. Lateral Bending

In addition to manual palpation and radiographic evaluation, fusion was evaluated using a custom designed approach (Yamaguchi *et al.*, 2012). Briefly, explanted spines were placed in a custom designed device capable displacement controlled lateral bending. Spines were anchored at the L1/2 and the sacrum; L1/2 was moved to three specific positions: bent left (-1 cm), neutral (0 cm) and bent right (+1 cm). In each position, the spine was imaged using micro-CT (pixel size: 18 μ m, 70 kV, 1.0 mm Al filter), and converted to 3D models using Mimics software. Six degree-of-freedom 3D segmental movements (3 rotational and 3 translational) were analyzed via a validated Volume-Merge method (Ochia *et al.*, 2006).

5.6.7. Histology

Following sacrifice, the spines were excised from the animals along with surrounding soft tissue and fixed in 10% buffered formalin. Samples were decalcified for 4 days using Cal-EXII according to the manufacturer's instructions. Successful decalcification was verified through absence of calcified hard tissue as visualized by micro-CT. Decalcified samples were embedded in paraffin, sectioned and stained via hematoxylin-eosin (H& E).

5.7. Acknowledgements

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Chapter 5 is currently being prepared as a manuscript for publication.

5.8. Figures

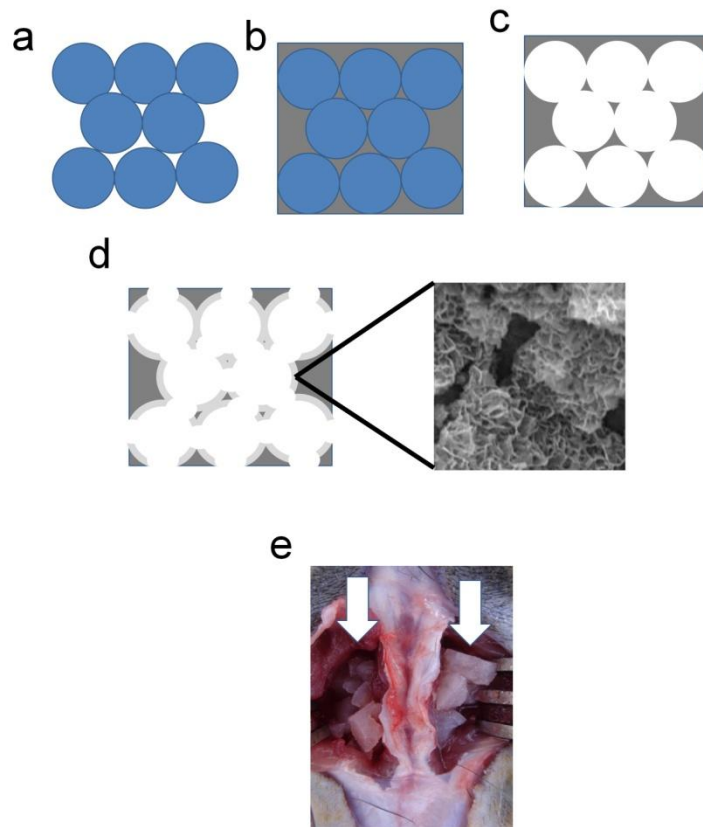


Figure 5.1. Schematic diagram of experimental procedures

Diagrammatic representation of the steps involved in the experiments, with (a) preparation of sintered template of PMMA microspheres (PMMA microspheres: blue spheres) (b) Polymerization of A6ACA-PEGDA hydrogel matrix (A6ACA-PEGDA: dark gray mass) around template (c) Solvent-leaching of PMMA template to obtain porous hydrogel matrices (d) Mineralization of porous hydrogels to obtain the synthetic biomineralized grafts (mineralized phase: light gray) and (e) implantation in nude rats for posterolateral fusion. In (d), the photograph shows the morphology of the mineralized phase on the pore surface. In (e), the white arrows depict the grafts placed on either side of the spine at L4-L5 following decortication of transverse processes.

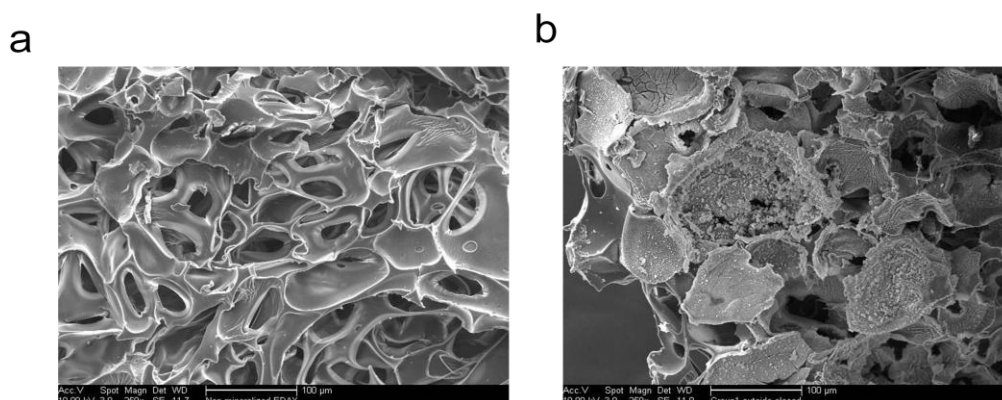


Figure 5.2. Microstructure of porous matrices

Scanning electron microscopy, showing morphology of porous A6ACA matrices (a) before and (b) after mineralization.

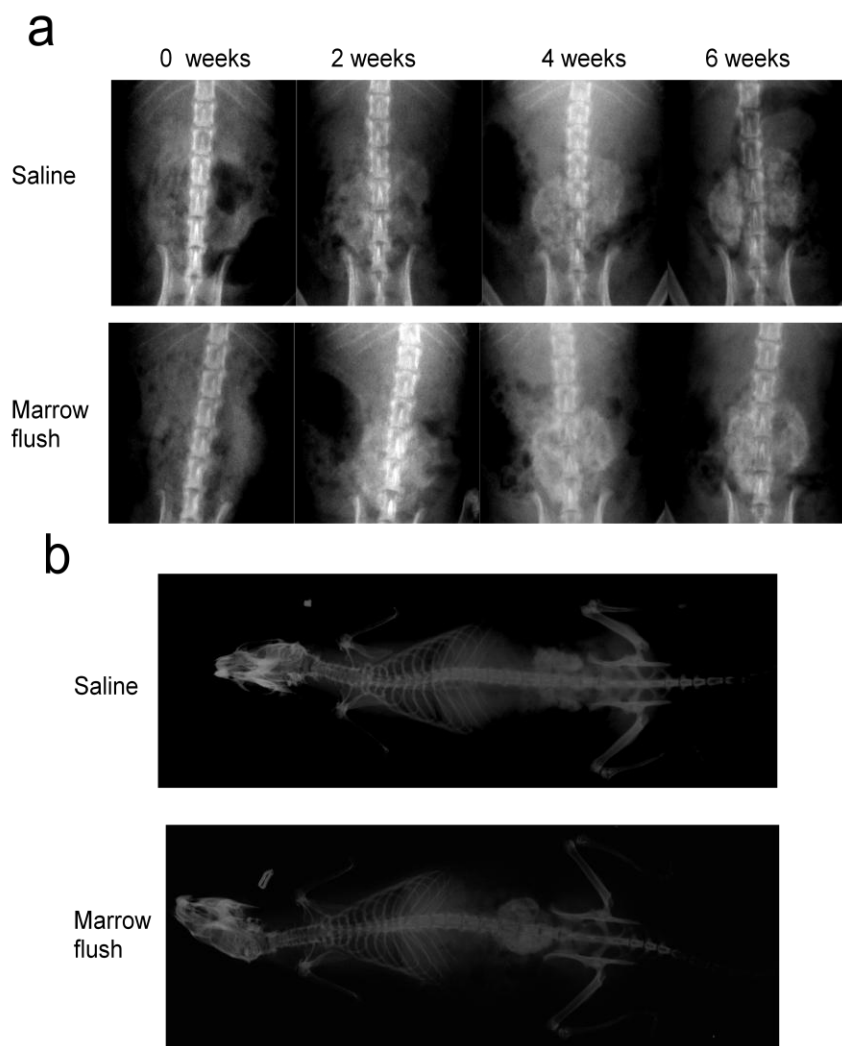


Figure 5.3. Radiographic evaluation of fusion

Evaluation of bone formation in representative animals through (a) digital live X-rays during the implantation period and (b) film-based X-rays collected immediately post-sacrifice at 8 weeks showing prominent fusion mass.

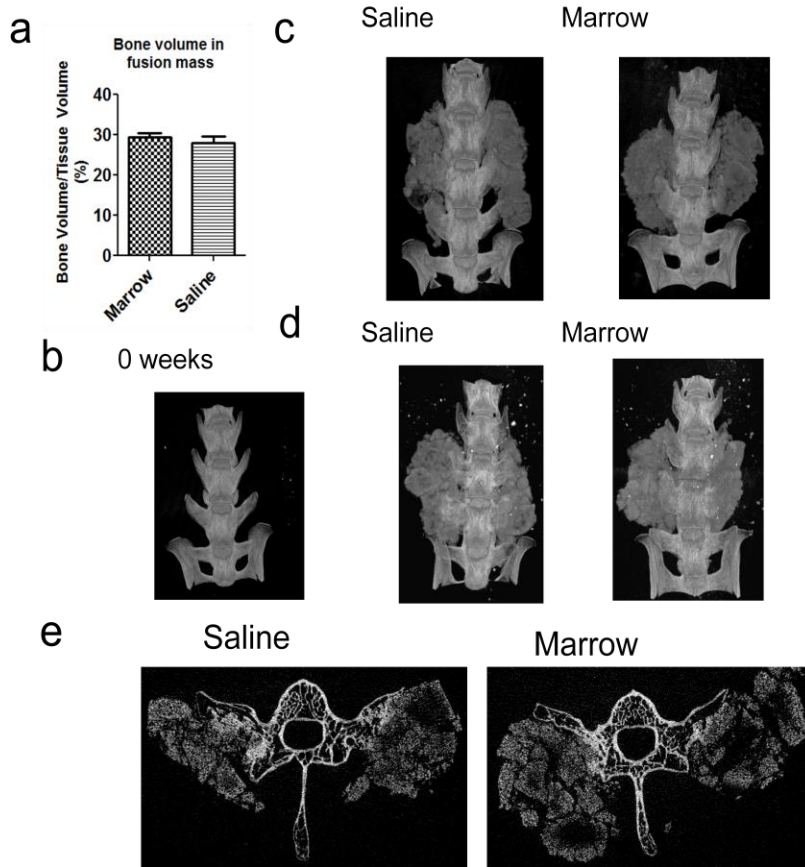


Figure 5.4. Micro-CT evaluation of fusion

(a) Bone volume in fusion mass for each group quantified through micro-CT. Error bars represent standard error of the mean. 3D micro-CT models generated (b) 0 weeks (c) 4 weeks and (d) 8 weeks after graft implantation. In (b), the white asterisks indicate the location of the implanted grafts; non-visibility of the grafts in the micro-CT at 0 weeks suggests the fusion masses at 4 and 8 weeks are due to new bone formation. (e) Transaxial section at 8 weeks, showing attachment of the transverse processes to the newly formed bone in both, the saline-soaked and marrow flush-soaked groups.

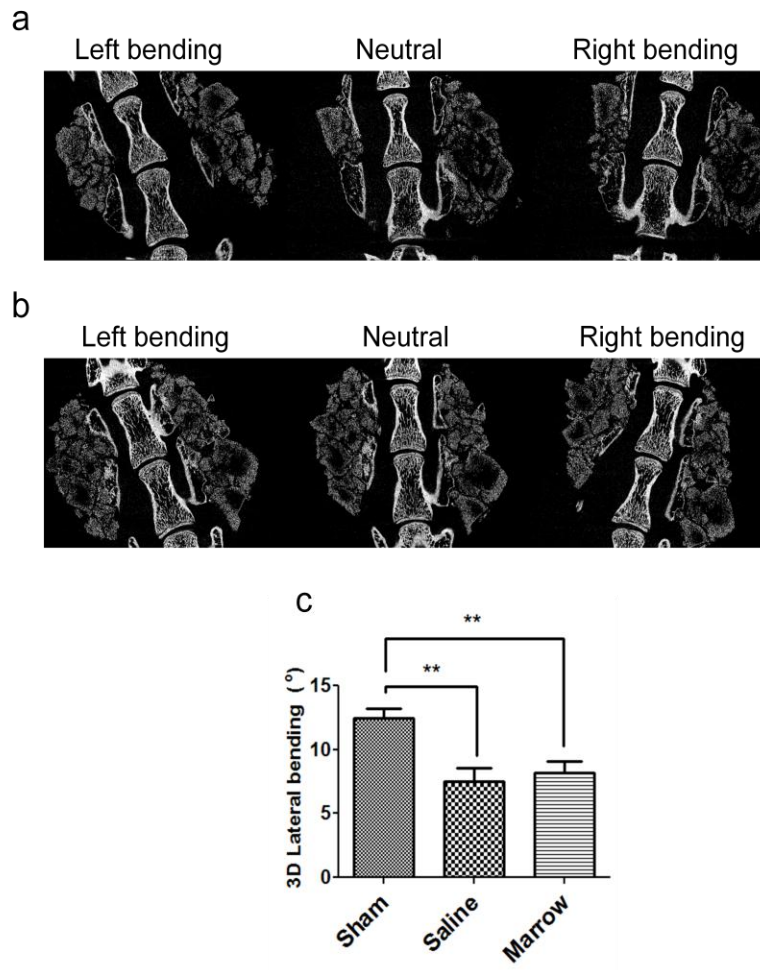


Figure 5.5. Lateral bending analysis of fused levels

Coronal views of lateral bending of (a) Saline-soaked (b) Marrow flush-soaked biomineralized grafts in the three bent positions, 8 weeks after implantation. (c) 3D lateral bending angles of saline-soaked and marrow-soaked grafts compared to sham surgery. Error bars indicate standard error of the mean (n=7, **: $p < 0.01$).

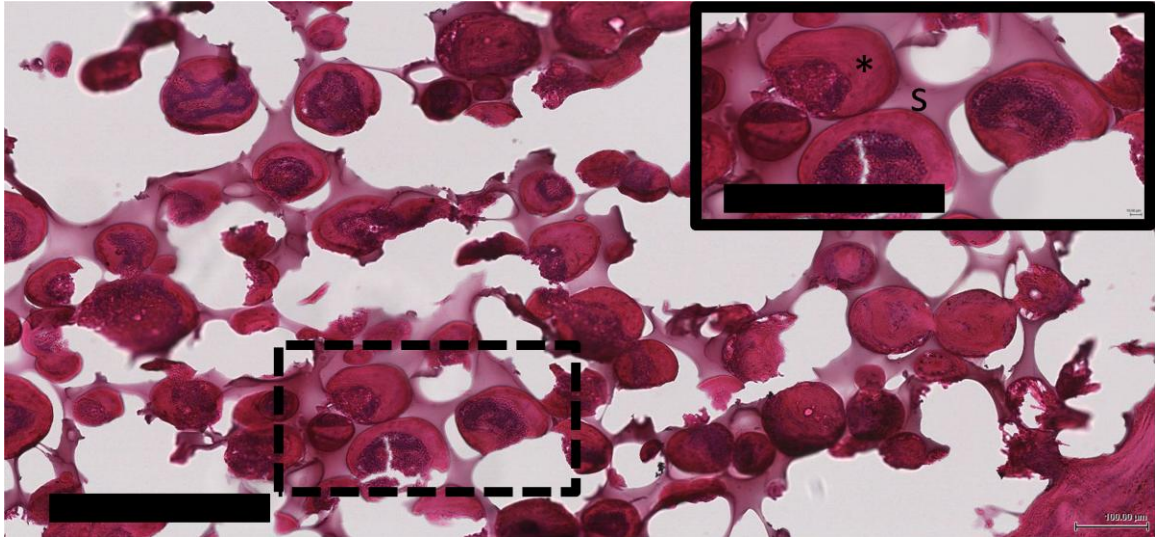


Figure 5.6. Histology

H & E staining of decalcified sections from the fusion mass of saline-soaked graft with a magnified image (inset) of the region highlighted with the dotted border. Scale bar in the main image represents 300 μm ; in the inset image, '*' represents bone, 's' represents the scaffold and the scale bar represents 200 μm

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CHAPTER 6: Rapid self-healing hydrogels

6.1. Abstract

Synthetic materials that are capable of autonomous healing upon damage are being developed at a rapid pace because of their many potential applications. Despite these advancements, achieving self-healing in permanently cross-linked hydrogels has remained elusive because of the presence of water and irreversible cross-links. Here, we demonstrate that permanently cross-linked hydrogels can be engineered to exhibit self-healing in an aqueous environment. We achieve this feature by arming the hydrogel network with flexible-pendant side chains carrying an optimal balance of hydrophilic and hydrophobic moieties that allows the side chains to mediate hydrogen bonds across the hydrogel interfaces with minimal steric hindrance and hydrophobic collapse. The self-healing reported here is rapid, occurring within seconds of the insertion of a crack into the hydrogel or juxtaposition of two separate hydrogel pieces. The healing is reversible and can be switched on and off via changes in pH, allowing external control over the healing process. Moreover, the hydrogels can sustain multiple cycles of healing and separation without compromising their mechanical properties and healing kinetics. Beyond revealing how secondary interactions could be harnessed to introduce new functions to chemically cross-linked polymeric systems, we also demonstrate various potential applications of such easy-to-synthesize, smart, self-healing hydrogels.

6.2. Introduction

Recent years have witnessed an increasing interest in the development of “smart” materials that can sense changes in their environment and can accordingly adapt their properties and function, similar to living systems. Over the last decade, we have discovered and demonstrated a class of smart hydrogels that exhibit unique biomimicking functions: thermoresponsive volume phase transitions similar to sea cucumbers (Varghese *et al.*, 2000), self-organization into core-shell hollow structures similar to coconuts (Varghese *et al.*, 2001), shape memory as exhibited by living organisms (Varghese *et al.*, 2001), and metal ion-mediated cementing similar to marine mussels (Varghese *et al.*, 2006). A common thread connecting these smart hydrogels is their possession of a unique balance of hydrophilic and hydrophobic interactions that endows the hydrogels with the biomimicking properties described above. In this study, we demonstrate how this concept of balancing hydrophilic and hydrophobic forces could be exploited to design chemically cross-linked hydrogels with self-healing abilities.

Indeed, materials capable of autonomous healing upon damage have numerous potential applications (Hager *et al.*, 2010; Messersmith *et al.*, 2007; Wojtecki *et al.*, 2011). So far, self-healing has been demonstrated in linear polymers (de Gennes, 1971), supramolecular networks (Burnworth *et al.*, 2011; Cordier *et al.*, 2008), dendrimer-clay systems (Wang *et al.*, 2010), metal ion-polymer systems (Holten-Andersen *et al.*, 2011; Lee *et al.*, 2011), and multicomponent systems (Chen *et al.*, 2002; Cho *et al.*, 2008; Ghosh and Urban, 2009; Kolmakov *et al.*, 2010; Toohey *et al.*, 2007). Whereas multicomponent thermosetting systems harness the ability of embedded chemical agents to repair cracks, supramolecular networks and noncovalent hydrogels employ secondary

interactions such as hydrogen bonding, ionic interactions, and hydrophobic association for healing. However, self-healing of permanently cross-linked systems such as hydrogels has remained elusive because of the presence of water and irreversible chemical cross-links, in spite of the many applications in biomedical sciences that such aqueous healing systems could offer.

We propose that self-healing could be achieved in hydrogels by decorating the polymer network with dangling hydrocarbon side chains containing polar functional groups that would mediate hydrogen bonding across two separate hydrogel pieces or across a rupture in the hydrogel. However, to achieve efficient and robust healing, the side chains must be sufficiently long and flexible, and the network sufficiently deformable, to make the functional groups across the interface accessible to each other beyond the corrugation of the interface. At the same time, the side chains should be short enough to minimize steric hindrance of the interacting functional groups and to prevent hydrophobic collapse of the side chains. In effect, the side chains should possess an optimal balance of hydrophobic and hydrophilic moieties.

We have previously shown that polymer hydrogels formulated from acryloyl-6-aminocaproic acid (A6ACA) precursors possess an optimal balance of hydrophobic and hydrophilic interactions that allows its side chains to bind to exogenous metal ions (Varghese *et al.*, 2006; Varghese *et al.*, 2001) and to extracellular proteins (Ayala *et al.*, 2011). The above finding suggests that the elastomeric properties of the A6ACA hydrogels along with their flexible side chains might be able to mediate hydrogen bonding across two hydrogel interfaces through the amide and carboxylic functional

groups. Consequently, we hypothesize that hydrogels synthesized from such precursors might exhibit self-healing in an aqueous environment in spite of their irreversible cross-linked architecture.

6.3. Results

6.3.1. A6ACA Hydrogels Demonstrate Rapid and Robust Self-Healing.

The A6ACA hydrogels were synthesized as described in Experimental Section (Figs. 6.1a., 6.S1, 6.S2 and SI text). We observed that two lightly cross-linked A6ACA hydrogels weld rapidly to each other within 2 s when brought in contact in low-pH aqueous solution ($\text{pH} \leq 3$) (Figure 6.1b) thus supporting our hypothesis. The healed hydrogels exhibited a strong interface capable of withstanding their own weight(s), repeated stretching, and exposure to boiling water (Figure 6.1c). The healed samples were able to sustain large deformations and recover their size and shape when the stress is released. This pH-mediated healing was also reversible: Two healed hydrogels separated when exposed to high pH (Figure 6.1d). The separated hydrogels were able to reheel upon reintroduction into a low-pH environment (Figure 6.1d). The cycle of healing, separation, and rehealing was repeated many (> 12) times without hysteresis; the healing occurred on the same timescale and with comparable weld-line strength as that of the original hydrogels.

6.3.2. Role of Hydrogen Bonding in Self-Healing

To confirm that the observed healing in A6ACA hydrogels was mediated through hydrogen bonding, we immersed the healed hydrogels into a urea solution, which is known to disrupt hydrogen bonds (McQueen-Mason and Cosgrove, 1994). As expected,

the immersion resulted in the separation of the two hydrogels at their interface (Figure 6.S3).

The role of hydrogen bonding was further analyzed by using FTIR–attenuated total reflectance (ATR) and Raman spectroscopy (Figure 6.2a,b, Table 6.1 and SI Text). The hydrogen-bonded terminal carboxylic-acid group was evident from the Raman band at $1,714\text{ cm}^{-1}$ and IR band at $1,704\text{ cm}^{-1}$ observed in healed hydrogels (Figure 6.2a,b). The spectroscopic analyses of the healed hydrogels suggested two different types of hydrogen bonding across the interface. First, the spectral features supported direct interaction of carboxyl groups with the amide groups of the opposing pendant side chain in an interleaved configuration (Figure 6.2c). In particular, the prominent IR band at $1,627\text{ cm}^{-1}$ and the corresponding weak Raman band at $1,624\text{ cm}^{-1}$, assigned to the amide I mode (majority C = O stretch, some C–N stretch), indicated the presence of strongly hydrogen-bonded amide groups (Barth and Zscherp, 2002; Colthup *et al.*, 1975). Second, the spectral features suggested a smaller fraction of carboxyl groups interacting with the opposing carboxyl groups in a face-on configuration (Dong *et al.*, 1997) (Figure 6.2c). Consistent with this configuration, we observed evidence of a small population of amide groups having similar amide I band intensity for healed and unhealed hydrogels, suggesting similarity in their H-bond environment irrespective of their protonation state. We confirmed through molecular modeling that the interleaved and face-on configurations are sterically feasible (Figure 6.S4).

The above analyses suggested an intriguing mechanism for the observed pH-mediated self-healing. At low pH, the terminal-carboxyl groups are mostly protonated, which allows them to form hydrogen bonds with other terminal-carboxyl groups or amide

groups across the interface, thereby allowing the hydrogels to weld (Figure 6.2c). At pH above their pK_a (4.4 for 6-aminocaproic acid, the parent amino acid from which the A6ACA monomer is synthesized) (Phadke *et al.*, 2010), the A6ACA carboxyl groups are deprotonated and exhibit significant electrostatic repulsion, which prevents hydrogen bonding (Figure 6.2d). We also found that the healing ability of the hydrogels diminished with prolonged exposure to low-pH environment prior to healing, but could be restored by immersing the hydrogels in a high-pH environment followed by reintroduction into a low-pH environment. The prolonged exposure of the hydrogels to a low-pH environment could lead to intramolecular hydrogen bonding, which decreases their availability to form intermolecular hydrogen bonds across the interface.

6.3.3. Mechanical Characterization of Healed Hydrogels.

A study of the temporal dependence of the healing indicated an increase in weld-line strength with time over a period of 10 s to 24 h (Figure 6.3a). Hydrogels healed for 10 s withstood more than 2.04 ± 0.07 kPa stresses whereas those healed for over 5 min failed upon an application of 2.7 ± 0.2 kPa stress. In both cases, the hydrogels always ruptured in the bulk region, whereas the welded interface remained intact (Figure 6.S5a), indicating a strongly healed interface. The low mechanical strength of the bulk region was attributed to its inherent soft nature compared to the surfaces that were in contact with the low-pH solution, as schematically shown in Figure 6.2e. Therefore, the interfacial region toughened as a result of protonation of the carboxyl groups and subsequent increase in their hydrogen bonding. In contrast, the interior bulk regions remained soft because due to inability of protons to diffuse into the polymer network

within the experimental timescales. However, after extended exposure (approximately 24 h) to low-pH solution, the hydrogels became capable of withstanding large stresses (35 ± 3 kPa) and broke at the interface. Moreover, the 24-h healed hydrogels became opaque because of protonation-induced hydrophobic collapse of the polymer chains (Figure 6.S5b).

Figure 6.3b shows that the maximum stress required to break 24-h healed hydrogels is $66 \pm 7\%$ of that of single hydrogel pieces of similar dimensions treated under identical conditions. The fracture stress in healed hydrogels was lower than in single hydrogels because failure in healed hydrogels involved only breakage of intermolecular hydrogen bonds across the interface whereas failure in single hydrogels involved breakage of both covalent bonds and intramolecular hydrogen bonds. The ratio of the elastic moduli, $E_{\text{healed}}/E_{\text{single}} = 1.1 \pm 0.5$ (where E_{healed} and E_{single} represent the elastic moduli of the 24-h healed and unhealed hydrogels, respectively) indicated little change in the stiffness of the hydrogels after healing.

6.3.4. Effect of Cross-Link Density and Side- Chain Length on Healing.

To determine the effect of cross-link density on healing, A6ACA hydrogels with varying cross-linker content were prepared (Figure 6.S2c). The self-healing depended strongly on the extent of cross-linking and thereby the swelling behavior of the hydrogels. Specifically, the interfacial strength of healed hydrogels decreased with increasing cross-linker content (Figure 6.3c). The reduction in healing efficiency could be attributed to either the restricted mobility of the side chains or to the decrease in the compliance of the hydrogel with increasing cross-linking, both of which could impede

the formation of hydrogen bonds across the interface. The latter effect, however, seems to be the more likely explanation given that the hydrogel still exhibits significant swelling at the high cross-link densities, indicating that the molecular pores might be considerably larger than the side chains and thus do not interfere significantly with the side-chain mobility.

Next, we investigated the effect of pendant side-chain length on healing by synthesizing hydrogels with similar cross-linker content but varying side-chain lengths, containing 1–10 methylene groups, terminating with a carboxyl group (Figure 6.S6a). Hydrogels with side chains containing 1–3 and 10 methylene groups did not exhibit any healing and those containing 7 methylene groups [*N*-acryloyl 8-aminocaprylic acid (A8ACA)] showed weak healing (Figure 6.S6b). The A8ACA hydrogels required more than 5 min to heal, and the healed hydrogels could be separated easily by a small stress (0.267 ± 0.008 kPa). Thus, interestingly, the healing ability depended nonmonotonically on the side-chain length.

The low healing ability of hydrogels with short side chains could be attributed to the limited “reach” of the carboxyl groups in mediating hydrogen bonds with functional groups across the interface, especially given that the hydrogel surfaces are likely corrugated. As the side chains become longer, the terminal-carboxyl groups become more flexible and increase their reach for hydrogen bonding, especially with the internal amide groups of the apposing hydrogel. When the side chains become too long, they begin to pose a larger steric hindrance to the interactions between the carboxyl and amide groups. In addition, the long side chains tend to aggregate and collapse because of increased hydrophobic interactions. This effect can be gleaned from the water solubility of

carboxylic acids of varying hydrocarbon chain lengths (Lide, 2009) (Figure 6.4a); i.e., chains containing more than six CH₂ groups become insoluble in water at concentrations similar to the effective concentration of side chains present in the hydrogel (approximately 0.02 M for A6ACA). Both the steric hindrance and hydrophobic collapse reduce the accessibility of the amide groups, leading to a reduction in the healing efficiency.

To confirm the suggested decrease in the accessibility of the amide groups with increasing chain length, we conducted molecular dynamics simulations of A6ACA, A8ACA, and *N*-acryloyl 11-aminoundecanoic acid (A11AUA) hydrogel networks in an aqueous medium (Figure 6.4b). We quantified the accessibility of the terminal-carboxyl and internal-amide groups in terms of the average number of hydrogen bonds they formed with the surrounding water molecules during the simulation (Figure 6.4c). Our simulations demonstrated a substantial decrease in the accessibility of the amide groups with increasing side-chain length, whereas the accessibility of the carboxyl groups changed only slightly with the chain length. Figure 6.4d shows representative configurations of the A6ACA and A11AUA hydrogel within one unit cell obtained from our simulations. The configurations are shown in a solvent excluded surface representation to illustrate the reduction in the accessibility of the amide groups (shown in blue) in going from the short to long side chains. The correlation between amide groups' accessibility and healing ability for A6ACA, A8ACA, and A11AUA hydrogel provided further support for the dominant role played by the interleaved hydrogen bonding configuration in self-healing as evidenced from spectroscopic analyses.

The observed dependence of healing on the side-chain length thus confirmed our hypothesis that self-healing is best exhibited by hydrogels possessing a balance of hydrophobic and hydrophilic interactions. Interestingly, this requirement along with that for flexible side chains to mediate hydrogen bonding across the interface explains why many polymeric systems including protein hydrogels do not exhibit robust self-healing despite their possessing amide and carboxylic functional groups.

6.3.5. Demonstrated Applications of Self-Healing Hydrogels.

The self-healing hydrogels developed here—which remain healed over a wide range of temperatures, light conditions, and humidity—could have numerous applications in medicine, environmental science, and industry. We explored several such applications.

We first investigated the application of these hydrogels as self-repairing coatings and sealants. We coated various surfaces with A6ACA hydrogels and mechanically damaged the coatings with 300- μm -wide cracks (Figure 6.5a). The coatings healed the imparted crack within seconds upon exposure to low-pH buffers (Figure 6.5b). Because this healing only requires initial contact, one can achieve repair by simply spraying the cracks with a low-pH buffer. We found that these hydrogels could adhere to various plastics like polypropylene and polystyrene even in their hydrated state; this is likely because of hydrophobic interactions (Figure 6.5c). This finding, in conjunction with the observed rapid pH-dependent healing, suggests that these hydrogels could be used as sealants for vessels containing corrosive acids. As a proof-of-concept, we created a hole in a polypropylene container, then coated it with A6ACA hydrogel, and finally poured

hydrochloric acid into it. The hydrogel instantly sealed the hole and prevented any leakage of the acid (Figure 6.5d).

We have also investigated the application of A6ACA hydrogels as tissue adhesives, with an emphasis on gastric tissue that is typically exposed to low pH, an environment in which the hydrogels can heal easily. The mucoadhesive ability of A6ACA hydrogels was investigated by using fresh gastric mucosa of rabbits. Figure 6.5e demonstrates that A6ACA hydrogels adhere well to the gastric mucosa and that the adhesion is strong enough to support the weight of the hydrogel. Thus, A6ACA hydrogels could indeed be used as tissue adhesives for stomach perforations, where the lightly cross-linked hydrogels could be injected to prevent leakage of gastric acids. In addition, such mucoadhesive hydrogels could also be employed for drug delivery if the hydrogels could store and release bioactive molecules without compromising their activity. To explore the potential of A6ACA hydrogels as drug carriers, we used tetracycline as a model system. The tetracycline-loaded hydrogels were exposed to a simulated gastric acid environment (pH 1.5) and the drug-release profile was evaluated. Tetracycline was released at a constant rate for 4 d after the initial bolus release (Figure 6.5f).

Finally, the ability of these hydrogels to fuse could also allow for the development of soft structures with complex architectures (Figure 6.S7). Such structures could find applications as soft actuators and in robotic devices.

6.4. Conclusions

In summary, we have demonstrated that self-healing can be achieved in chemically cross-linked systems through introduction of pendant side chains possessing an optimal balance of hydrophilic and hydrophobic moieties, using A6ACA as a model system. The self-healing hydrogels described here represent an exciting class of smart, easy-to-synthesize materials with widespread potential applications in biology, medicine, and engineering.

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6.6. Experimental section

6.6.1. Synthesis of acryloyl amino acid monomers

The monomers were synthesized and characterized as described elsewhere. See Supplementary Text for more details.

6.6.2. Synthesis and characterization of hydrogels

Hydrogels were prepared by free radical polymerization. See Supplementary Text for more details on synthesis and characterization of hydrogels.

6.6.3. Healing of the hydrogels

Healing of hydrogels was carried in different buffer solutions with pH ranging from 0.3-7.4. Specifically, we used 0.5 M hydrochloric acid (pH 0.3), 1x phosphate-buffered saline (pH 7.4) and other buffer solutions as detailed in Table 6.2 S2. The hydrogel samples were brought into contact with each other without application of any external force. For ease of visualization, the hydrogels were dyed yellow and maroon by soaking them in PBS containing 0.5% (vol/vol) methyl red indicator and approximately 0.002% (wt/vol) alizarin red S, respectively.

6.6.4. Mechanical characterization

Butt-welded hydrogels were used for mechanical measurements. To determine the interfacial strength of the hydrogels healed for 10 s and 5 min, we used a custom-designed approach where known weights were applied to healed hydrogels and the resulting engineering stress required to break the healed hydrogels was calculated. The mechanical properties of 24 h-healed hydrogels were determined using an Instron 332

Universal Testing System (Instron) equipped with a Model 2519-104 force transducer. A load cell of 450 N was fitted to the instrument and tensile tests were done at cross-head speed of 15 mm/min. The data acquisition and processing were performed with BlueHill software. The tensile modulus was determined by calculating the slope of the linear region of the stress-strain curve, whereas the fracture stress was determined from the peak of the curve.

6.6.5. Reversibility of Healing

Cylindrical hydrogels were healed via butt welding as described above, and then immersed in 1 M NaOH (pH 14) for 10 min for separation. The separated hydrogels were then briefly rinsed in PBS to remove excess NaOH and reintroduced into an acidic solution (pH 0.3) and healed by maintaining the surfaces in contact for less than 5 s. These rehealed hydrogels were then reintroduced into 1 M NaOH solution for separation. This cycle of healing-separation-rehealing was performed more than 12 times to test the reversibility of healing. Separation of healed hydrogels was also examined in a standard buffer solution of pH 10 (Fisher Scientific, Inc) and it was found to be at a slower rate compared to those separated at pH 14.

6.6.6. Stability of healed hydrogels in water and effect of temperature

The completely healed hydrogels were immersed in DI water for more than a month to determine their stability at ambient temperature. To determine the effect of temperature on the stability, the healed hydrogels were immersed in boiling water at 100 °C for 1 h.

6.6.7. Effect of Urea on healing efficacy

To investigate the contribution of hydrogen bonding on healing, the butt-welded hydrogels were immersed in excess of a 30% (wt/vol) solution of urea in DI water. Another healed hydrogel immersed in DI water was used as a control.

6.6.8. FTIR-ATR and Raman Spectroscopy

Spectroscopic analysis was carried on loosely cross-linked A6ACA hydrogels that were healed in 0.5 M HCl for 24 h, along with unhealed hydrogels (pH 7.4) for comparison. The healed and unhealed hydrogels were dried for 24 h at 37 °C prior to performing Raman and FTIR–ATR spectroscopy to minimize interference of hydrogen-bonded water molecules. The FTIR spectra from 4,400 to 600 cm^{-1} were acquired with a Perkin Elmer Spectrum RX Fourier transform infrared spectrometer. Samples were placed on the diamond window of a PIKE MIRacle ATR attachment. Each reported spectrum is the average of four scans, and the resolution is 2 cm^{-1} . Raman spectroscopy was performed with a homebuilt Raman microscope system. A mixed-gas Kr-Ar ion laser (Coherent Innova 70C) provided continuous-wave excitation at 514.5 nm. The beam was sent through a 514.5-nm interference filter (Semrock) and directed into a modified fluorescence alignment port of a Zeiss Axio Imager A1m upright microscope. A broadband beam splitter (Edmund Optics) directed a small portion (approximately 10%) of the beam downward to the entrance aperture of a 50 $\times$ objective. The power at the sample was 5.2 mW. Back-scattered light was collected and collimated with the same objective, filtered with a 514.5-nm edge filter (Semrock), and focused on the entrance slit of a 0.32-m focal length spectrograph (Horiba Jobin Yvon; iHR-320). Raman scattered

light was dispersed with a 1,200 grooves/mm-ruled grating and detected by a thermoelectrically cooled open-electrode CCD detector (Horiba Jobin Yvon Synapse). Wavelength calibration was performed using known lines of Hg/Ar and Ne lamps for windows centered at 550 and 610 nm, respectively. See Supplementary Text for more details.

6.7. Supplementary Text

6.7.1. Monomer synthesis and characterization

Monomers N-acryloyl 2-glycine (A2AGA), N-acryloyl 4-aminobutyric acid (A4ABA), N-acryloyl 6-aminocaproic acid (A6ACA), N-acryloyl 8-aminocaprylic acid (A8ACA), and N-acryloyl 11-aminoundecanoic acid (A11AUA) were synthesized from glycine (Fisher Scientific Inc), 4-aminobutyric acid, 6-aminocaproic acid, 8-aminocaprylic acid (Acros Organics Inc.) and 11-aminoundecanoic acid (Aldrich), respectively, as described elsewhere (Ayala *et al.*, 2011). Briefly, for A2AGA, 0.1-mol glycine and 0.11-mol NaOH were dissolved in 80 ml deionized water in ice bath under vigorous stirring. To this, 0.11-mol acryloyl chloride in 15 ml tetrahydrofuran was added dropwise. The pH was maintained at 7.5-7.8 until the reaction was complete. The reaction mixture was then extracted with ethyl acetate. The clear aqueous layer was acidified to pH 2.0 and then extracted again with ethyl acetate. The organic layers were collected, combined, and dried over sodium sulfate. The solution was then filtered, concentrated, and precipitated in petroleum ether. Further purification was achieved by repeated precipitation and the product was lyophilized. Synthesis of other monomers followed similar procedure, with variations in pH during the acidification: pH 2.0 for

A4ABA; pH 3.0 for A6ACA; and pH 5.0 for A8ACA and A11AUA. Proton nuclear magnetic resonance spectra (^1H NMR) of monomers were recorded with a Varian Mercury-400 spectrometer at 400 MHz. Carbon-13 nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a Varian Mercury-400 spectrometer at 100 MHz; CDCl_3 or D_2O were used as solvents (Ayala *et al.*, 2011).

6.7.2. Synthesis of hydrogels

Hydrogels were prepared by free radical polymerization in aqueous solution containing 1 mmol/ml of monomer, N,N'-methylene bisacrylamide (Bis-Am), 0.5% ammonium persulfate (initiator), and 0.1% tetramethylethylenediamine (accelerator) (Figure 6.S1a). To synthesize A6ACA hydrogels containing different crosslinker content, 0.1%, 0.2% and 0.5% (w/v) BisAm (Sigma-Aldrich Inc) was added to the 1 M deprotonated A6ACA solution and polymerized as described above using the APS/TEMED redox initiators for 16 hours at 37°C. To create hydrogels with varying pendant side chains, we followed the same procedure. Specifically, 1 M solutions of the respective monomers (0.1291 g/ml for A2AGA, 0.157 g/ml for A4ABA, 0.185 g/ml for A6ACA, 0.213 g/ml for A8ACA and 0.241 g/ml for A11AUA) were deprotonated using equimolar NaOH and used.

Loosely crosslinked A6ACA hydrogels were prepared using high concentrations of A6ACA monomers via chain transfer (Figure 6.S1b). Previous studies have shown that alkyl monomers having long pendant side chains could lead to loosely crosslinked networks during radical polymerization as a consequence of chain transfer at high concentration of monomers (Lazar *et al.*, 2000). In this study, we adopted this concept to

create loosely crosslinked A6ACA hydrogels, where 1M A6ACA was dissolved in 1M sodium hydroxide to deprotonate the carboxyl groups of A6ACA. This solution was then polymerized using 0.5% ammonium persulfate (APS) as initiator and 0.15% tetramethylethylenediamine (TEMED) as accelerator in cylindrical polypropylene molds measuring 0.5 cm in diameter and 2.5 cm in length. Polymerization was allowed to proceed for 16 hours at 37°C. The as-synthesized hydrogels exhibited an intact swollen structure with an equilibrium swelling ratio of 56 ± 3 g/g in PBS (Figure 6.S2a).

The hydrogel formation was also characterized through ^{13}C NMR spectroscopy (Figure 6.S2b). The crosslinked hydrogels were dialyzed against DI water in a dialysis tube (MWCO=500 Da) for 48 hours and ground in a mortar into fine particles and then freeze-dried. The freeze-dried powder was swollen in D_2O for NMR analysis. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy was recorded on a Varian VX500 500MHz spectrometer. The NMR spectrum was compared against linear A6ACA polymer as described below. Linear poly(N-acryloyl 6-aminocaproic acid) was dissolved in D_2O at a concentration of 1 % (w/v); lightly crosslinked polyA6ACA was fully swollen in D_2O and transferred into an NMR tube before spectroscopic analysis. Figure 6.3b S2b shows that all peaks of the loosely crosslinked-A6ACA hydrogels (178.2, 36.1, 33.0, 24.9, 22.5, and 21.2 ppm) were identical or close to those in the spectrum of linear poly-A6ACA except for one peak at 67.0 ppm (labeled by an asterisk). This additional peak is attributed to chemical crosslinks formed during polymerization as a consequence of chain transfer at high monomer concentration (Britton *et al.*, 1998; Xu *et al.*, 2010).

6.7.3. Synthesis of linear A6ACA polymer

2 grams (10.8 mmol) of A6ACA, 0.432 grams (10.8 mmol) of NaOH and 7.8 mg (0.1 mmol) of 2-mercaptoethanol (chain transfer agent) were dissolved in 40 mL of DI water at room temperature. Upon complete dissolution of the reactants, 40 μ L of TEMED was added to the solution, and purged with argon for 30 minutes. 20 mg of APS in 2 mL of DI water was then added to the solution under argon purge. The solution was transferred to an oil bath at 40°C and reacted overnight. The polymer solution was cooled to room temperature and poured into 800 mL of acetone. The precipitate was collected and dried under vacuum at room temperature. The product was further purified by dialysis against DI water in a dialysis tube (MWCO=500 Da) for 48 hours and freeze-dried before analysis. The usage of 2-mercaptoethanol as a chain transfer agent avoids the transfer of free radical to the polymer backbone and thereby prevents subsequent cross-linking of the polymer (Xu *et al.*, 2010).

6.7.4. Hydrogel swelling ratio measurements

The hydrogels were immersed in excess of 1X phosphate-buffered saline (PBS; pH 7.4) for 48 hours following synthesis to allow equilibration with constant change of PBS. The hydrogels were weighed after equilibrium swelling to determine their wet weight and after subsequent freeze-drying to determine their dry weight. Swelling ratio was calculated as the ratio of wet to dry weight. Samples were prepared as triplicates and averages were calculated with standard deviation (Figure 6.S2c).

6.7.5. Spectroscopic analyses

Spectroscopic analysis was carried out on healed and un-healed A6ACA hydrogels. Samples were dried at 37°C for 24 hours prior to measurement of Raman and FTIR spectra. The Raman spectra of the healed and un-healed samples are shown in Figure 6.2a. The appearance of a band at 1409 cm^{-1} for unhealed hydrogels indicates the presence of carboxylate (COO^-) functional groups. This frequency is well within the typical range expected for the COO^- symmetric stretch; the corresponding band in the FT-IR spectrum is at 1403 cm^{-1} (Figure 6.2b) (Barth and Zscherp, 2002; Colthup *et al.*, 1975). The healed samples uniquely exhibits a strong Raman band at 1714 cm^{-1} (Figure 6.2a), which is best assigned to a hydrogen-bonded carboxylic acid group. The corresponding band in the FT-IR spectrum (Figure 6.2b) is at 1704 cm^{-1} . The close correspondence of the IR and Raman frequencies indicates that this pair of bands is unlikely to be a signature of a cyclic carboxylic acid dimer. A cyclic dimer generally has a Raman-active in-phase combination of C=O stretches that is expected to be downshifted to $1680\text{-}1640\text{ cm}^{-1}$ and an IR active out-of-phase combination located in the $1720\text{-}1680\text{ cm}^{-1}$ range (Colthup *et al.*, 1975). Furthermore, a cyclic carboxylic acid dimer would be expected to show other signatures of out-of-plane $\text{OH}\cdots\text{O}$ hydrogen deformation in the $960\text{-}875\text{ cm}^{-1}$ region of the FT-IR spectrum, and these bands are either weak or absent in healed protonated hydrogels (Colthup *et al.*, 1975). A reasonable hypothesis is that some of the protonated $-\text{COOH}$ groups reach the amide groups of opposing pendant chains, and form a pair of H-bonds (acceptor and donor) to the respective NH and C=O groups of the amide (interleaved configuration). The following spectral assignments are consistent with this configuration.

1. Raman and IR activity at $1714/1704\text{ cm}^{-1}$ is attributed to the C=O stretch of the carboxylic acid group that is hydrogen bonded to the NH of the amide.
2. The NH stretch at 3310 cm^{-1} is a typical frequency for an H-bonded N-H group.
3. A prominent band in the healed A6ACA hydrogel FT-IR spectrum at 1627 cm^{-1} and a corresponding weak band in the Raman spectrum at 1624 cm^{-1} are assigned to the amide I mode (majority C=O stretch, some C-N stretch). These frequencies are $\sim 18\text{ cm}^{-1}$ downshifted relative to the $1642\text{-}1643\text{ cm}^{-1}$ band that is assigned to the amide I at high pH. A downshift is expected for enhanced H-bonding to the amide group (Barth and Zscherp, 2002; Colthup *et al.*, 1975) such as provided by direct interaction between -COOH and amide groups. A similar shift for A6ACA was noted in earlier work at pH 5.5, however a spectrum at lower pH was not recorded and a strong band at 1628 cm^{-1} was not clearly assigned to carboxylic acid/amide interaction as we propose here (Barbucci *et al.*, 1989; Colthup *et al.*, 1975).

We note substantial amide I band intensity at $\sim 1645\text{ cm}^{-1}$ (FT-IR, shoulder) and 1642 cm^{-1} (weak Raman band) for healed hydrogels, which is nearly the same frequency as the amide I in unhealed hydrogels (Figure 6.2a,b and Table 6.1). This observation suggests that some population of amide groups has a similar H-bond environment at both pH extremes. For this population of interacting chains, the formation of interleaved pendant chains at low pH seems implausible. Instead, it is possible that some of the -COOH groups hydrogen bond with pairs of -COOH groups on opposing strands, in a manner that has previously been termed as “face-on” (Barth and Zscherp, 2002; Dong *et*

al., 1997). The amide groups of this subset of pendant chains would have similar interactions with each other (or with neighboring water molecule) at either high or low pH, which is consistent with the similar amide I frequencies at low and high pH. Furthermore, the fact that the face-on structure is less symmetric than a cyclic dimer is consistent with the overlapping IR and Raman frequencies.

6.7.6. Molecular dynamics simulations of hydrogel networks

To investigate the effect of side chain length on healing efficiency, we performed molecular dynamics simulations of hydrogel networks built from A6ACA, A8ACA and A11AUA monomers having side chains of lengths 5, 7, and 10 CH₂ groups, respectively. The 3D network structure of the hydrogel comprising of 20 monomers between each crosslink was assembled using the procedure of Jang *et al.* (Jang *et al.*, 2009). Our simulation box (unit cell) had dimensions of ~9.2 x 6.4 x 6.4 nm and it consisted of water molecules and a 9-arm network motif placed symmetrically inside the simulation box. This motif consisted of two 4-arm crossed junctions connected along the x direction by a chain of 20 monomers, where each arm was a chain of 10 monomers (see Figure 6.9a 4a, left). The motif was placed symmetrically inside the simulation box such that its arms in the y and z direction touched the faces of the simulation box while there was a 0.5 nm gap on either sides of the two junctions in the x direction. The simulation box, when replicated in all directions through periodic boundary conditions (PBCs), yielded the desired hydrogel network shown in Figure 6.4b, right. Specifically, implementation of PBCs allowed us to covalently connect the 8 arm ends to their periodic images in the y and z direction while the water filled gap in the $\pm x$ direction prevented continuity of the

network along the x direction and allowed the creation of a hydrogel-water interface in between periodic images of the network.

Due to computational issues, we are limited to examining high crosslink densities: the crosslinks in our network are separated by 20 monomers-long chains while those in the experimental system are more sparsely distributed (~ 150 monomers apart from rough calculations). However, because the side chains still remain significantly smaller than the molecular pores in the network, the side chain conformations and their accessibility for mediating external hydrogen bonds are not likely to be affected much by the larger crosslink density used in our simulations. Hence, our “compact” model of the hydrogel network might still yield quantitative information on the conformations of the side chains and their dependence on side chain length.

The intra- and intermolecular interactions in our network were described using the *ab initio*-based polymer consistent force field (PCFF) (Sun, 1998). All simulations were performed using the large-scale atomic/molecular massively parallel simulator (LAMMPS) package. We added ~ 6000 water molecules, treated using the TIP3P model, into the unit cells constructed for the networks studied here. The resulting configurations were energy minimized, equilibrated for 50 ps in the constant-volume-temperature (NVT) ensemble at 300 K, thermally annealed at 800 K for 40 ps, and then cooled back to 300 K. Further equilibration was performed in the constant-pressure-temperature (NPT) ensemble at 300 K and 1 atm. The time step was taken as 0.25 fs in all simulations and a Nose-Hoover thermostat was employed to keep temperature constant. The final

equilibrated density of equilibrated hydrogels at 300 K and 1 atm was calculated as 1.092 g/cm³ for A6ACA, 1.0744 g/cm³ for A8ACA and 1.065 g/cm³ for A11AUA.

To determine the accessibility of the carboxyl and amide groups of the network side chains for mediating hydrogen bonds, we picked 5 representative configurations of the network from 250 ps-long simulation runs. We quantified the accessibility of each group in terms of the number of hydrogen bonds it forms with the water molecules. We hypothesize that the accessibility of the functional groups for interacting with water molecules is a good measure for their accessibility for interacting with functional groups from the apposing hydrogel surface. The number of hydrogen bonds mediated by the amide and carboxylic groups of the chosen configurations were calculated by using UCSF Chimera software using a tolerance of 0.3 Å and 20° from the precise geometrical criteria for hydrogen bonding (Mills and Dean, 1996).

6.8. Figures

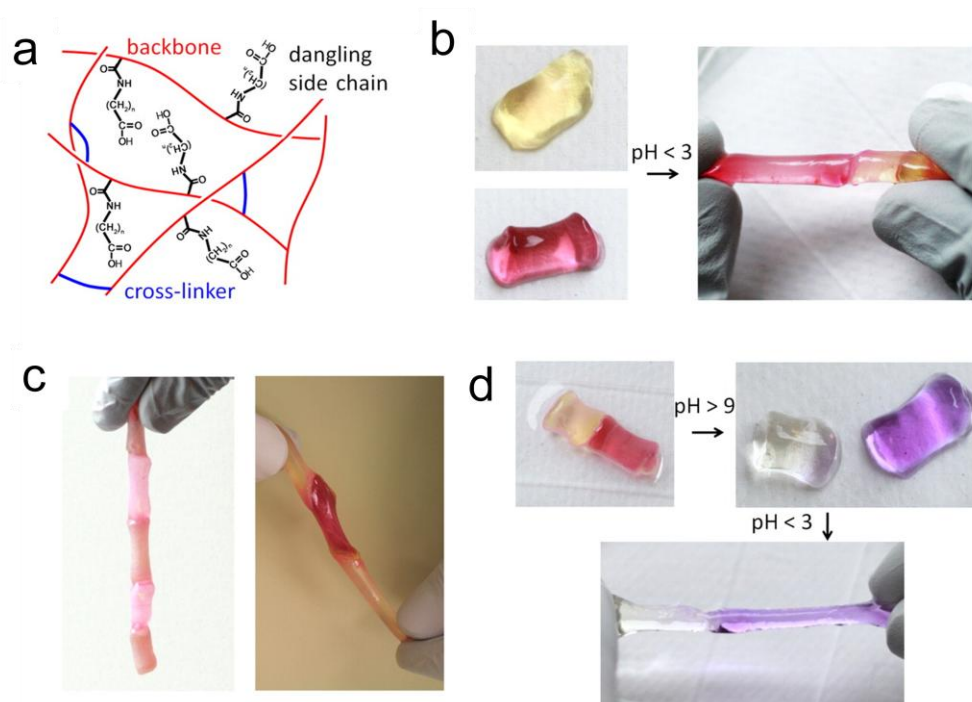


Figure 6.1. Self-healing hydrogels

(a) Schematic illustration of the structure of self-healing A6ACA hydrogels containing dangling side chains terminating with a carboxyl group. (b) deprotonated hydrogels at pH 7.4 (left) heal in low-pH solution ($\text{pH} \leq 3$) (right). The hydrogels are dyed yellow and maroon to allow for easily distinguished interface. (c) Healed hydrogels carrying their own weight(s) (left) and being stretched manually (right) illustrate the weld-line strength. (d) The healed hydrogels at low pH (left) separate after exposure to high pH solution ($\text{pH} > 9$) (right). The change in color is due to reaction of the dyes with the NaOH solution. (Lower) The separated hydrogels in (upper) re-heal upon exposure to acidic solution ($\text{pH} < 3$).

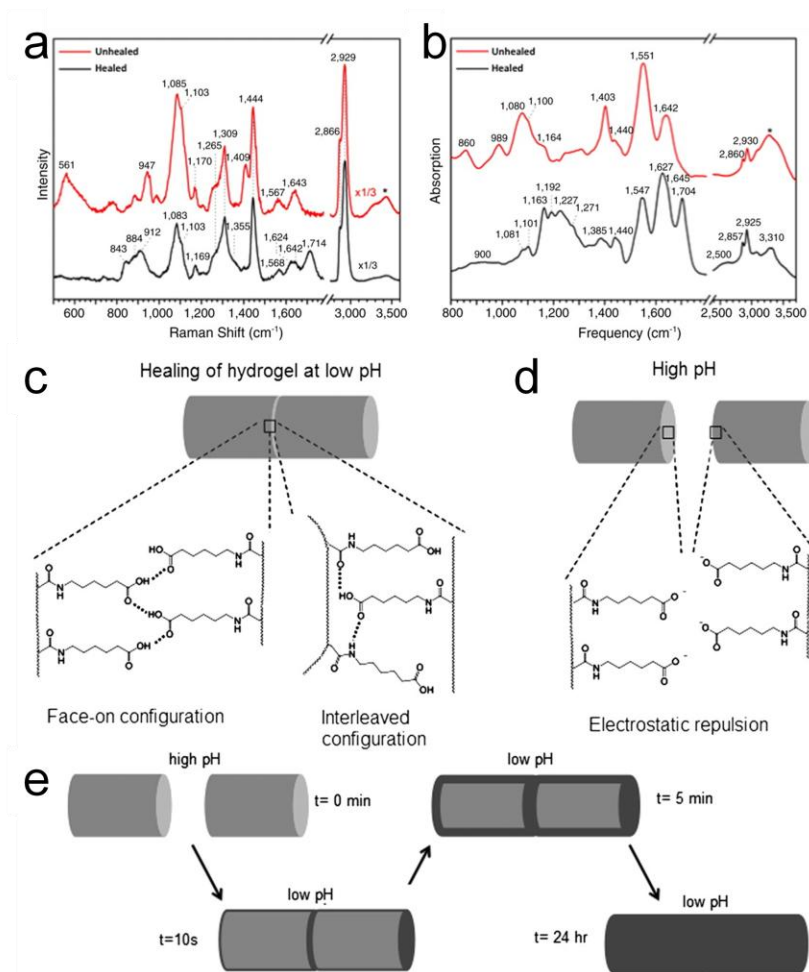


Figure 6.2. Mechanism of self-healing in A6ACA hydrogels

Raman (a) and FTIR-ATR (b) spectroscopy of healed (low pH) and unhealed (high pH) hydrogels demonstrating the presence of multiple types of hydrogen-bonded carboxyl groups. (c) Deduced molecular structures of pendant side chains in the face-on and interleaved hydrogen bonding configurations responsible for healing at low pH. (d) Structure of the pendant side chains in the unhealed hydrogels at high pH. At high pH, the carboxyl groups become deprotonated, leading to strong electrostatic repulsion between the apposing side chains, thus preventing healing. (e) Schematic explanation for why the healed hydrogels exhibit a mechanically stronger weld-line compared to the bulk after healing for small timescales, and vice versa at very longer times. Darker gray represents the toughened regions of the hydrogels due to protonation. The lighter gray represents the deprotonated (softer) regions of the hydrogels, which protonate and toughen with increasing exposure to low-pH solution.

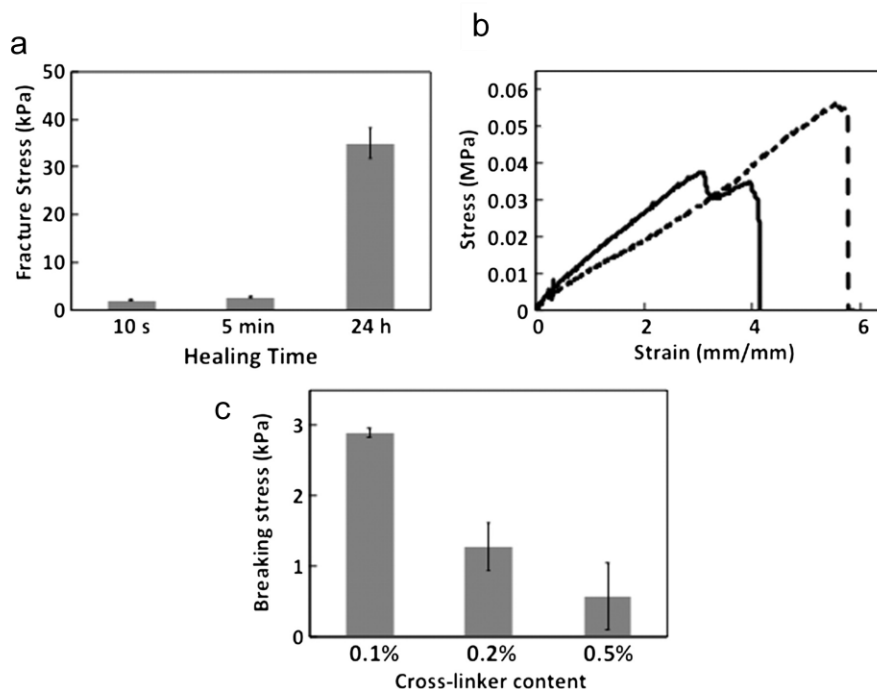


Figure 6.3. Mechanical characterization of healing and healed hydrogels.

(a) Effect of healing time on fracture stress. (b) Stress–strain curve, comparing tensile properties of 24-h healed gels with a single, unhealed hydrogel at identical conditions. The solid and dashed lines represent data from healed and unhealed hydrogels, respectively. (c) Fracture stress as a function of the extent of cross-linking for hydrogels containing 0.1%, 0.2%, and 0.5% of cross-linker (N, N'-methylenebisacrylamide) content. Error bars in a and c represent the standard deviation ($n = 3$).

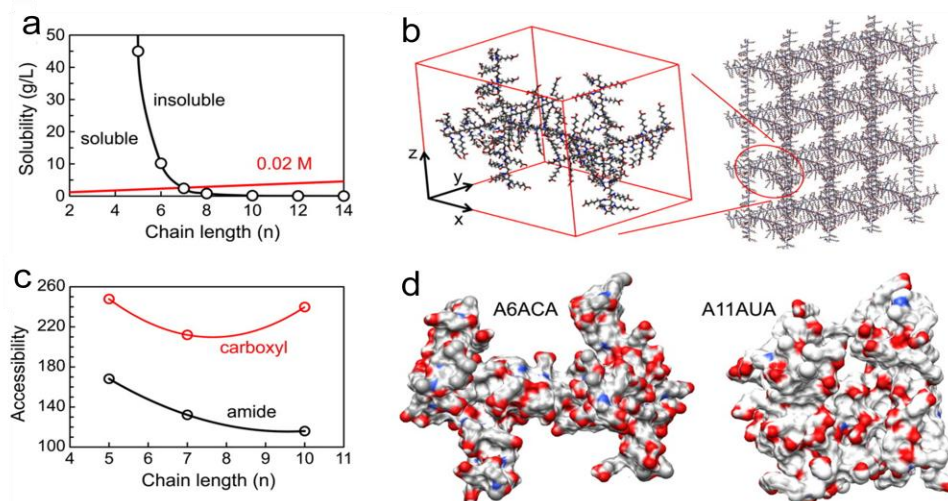


Figure 6.4. Effect of side-chain length on the accessibility of functional groups.

(a) Solubility of carboxylic acids of varying hydrocarbon chain lengths in water (black circles). Dashed red line indicates the density of carboxyl groups present in the hydrogels. (b) Molecular dynamics simulations setup for A6ACA network. A nine-arm motif of the network (left) is used to create the 3D network structure (right) via periodic boundary conditions. (c) Computed accessibilities of the amide and carboxyl groups in the A6ACA, A8ACA, and A11AUA hydrogels. (d) Representative configuration of the A6ACA and A11AUA network obtained from molecular dynamics simulations, shown in terms of solvent excluded surface, illustrating the higher accessibility of the amide groups in the former network. Blue, red, light gray, and white colors correspond to the surfaces of nitrogen, oxygen, carbon, and hydrogen, respectively. Chain length n in a and c represent number of CH_2 groups in the carboxylic acids and side chains, respectively.

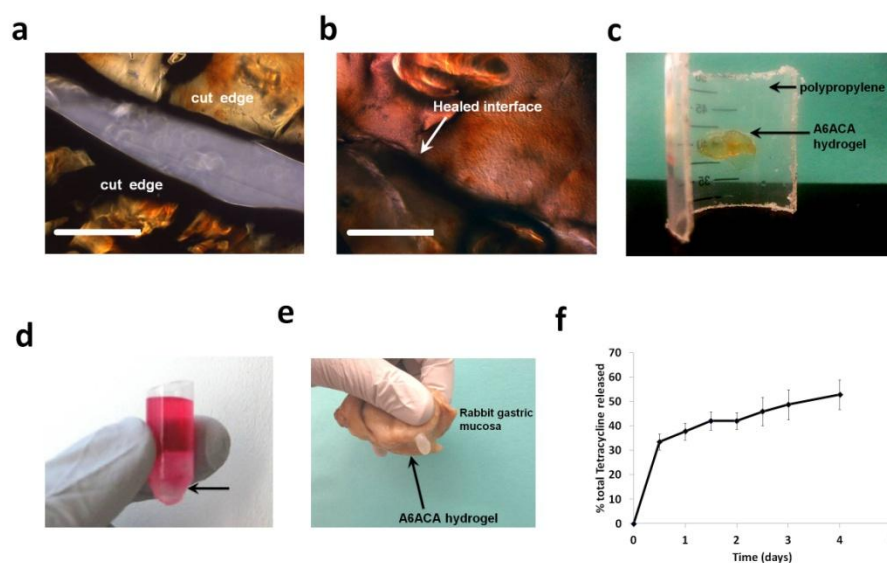


Figure 6.5. Applications of self-healing A6ACA hydrogels.

The rupture site within the A6ACA coating on a polystyrene surface (a) before and (b) after healing. The coating was colored using a dye for easy visualization and the observed color change after healing is caused by its exposure to low-pH buffer. (Scale bars: 500 μm). (c) Adhesion of A6ACA hydrogels to a poly(propylene) surface. (d) Polypropylene container holding acid solution after sealing the hole with A6ACA hydrogel. The arrow indicates the sealed site. (e) Adhesion of A6ACA hydrogels to rabbit gastric mucosa. (f) Cumulative tetracycline release from A6ACA hydrogels plotted as a function of time. Error bars represent standard deviation ($n = 4$).

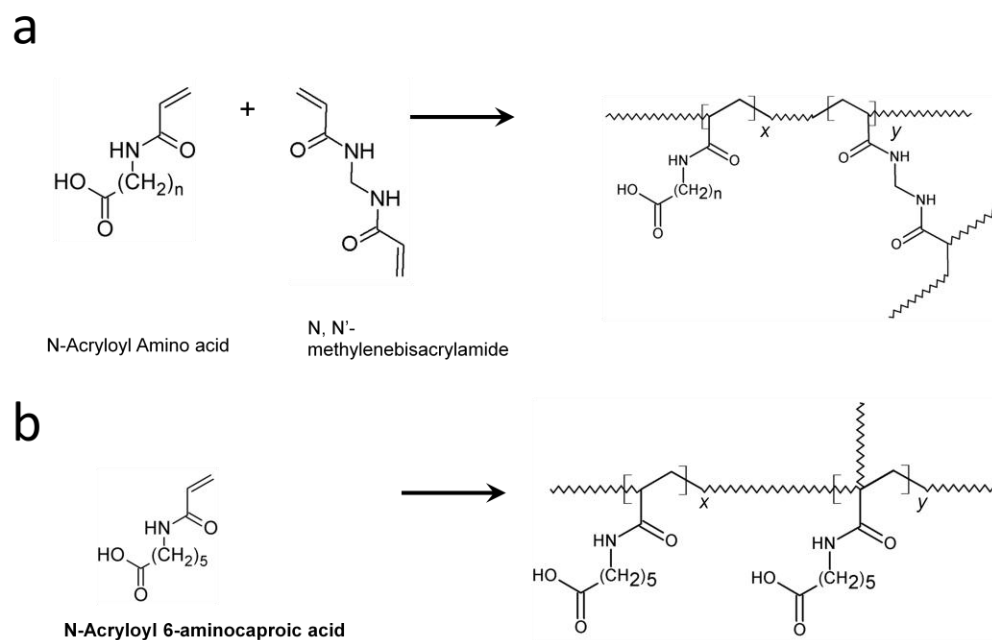


Figure 6.S1. Synthesis scheme for self-healing hydrogels

(a) Synthesis of N-acryloyl amino acid hydrogels with varying side chain lengths (A2AGA, $n=1$; 4ABA, $n=3$; A6ACA, $n=5$; A8ACA, $n=7$; and A11AUA, $n=11$) by using N,N'-methylenebisacrylamide as a cross-linker (b) Loosely cross-linked N-acryloyl 6-aminocaproic acid in the absence of N,N'-methylenebisacrylamide. In both schemes, $x \gg y$.

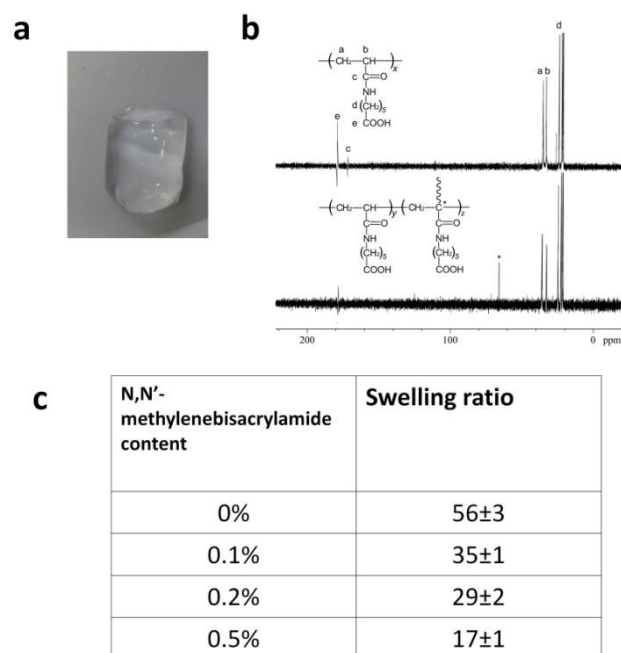


Figure 6.S2. Synthesis and characterization of A6ACA hydrogels

Equilibrium swollen, loosely cross-linked A6ACA hydrogel (a) and its ^{13}C NMR spectroscopic analysis (b). The top spectrum represents linear polyA6ACA whereas the bottom spectrum represents cross-linked A6ACA. All peaks of cross-linked A6ACA (178.2, 36.1, 33.0, 24.9, 22.5 and 21.2 ppm) are either identical or close to those of the linear polymer, except one peak at 67.0 ppm (labeled by the asterisk). This peak is attributed to the chemical cross-links formed during polymerization as a consequence of chain transfer at high monomer concentration. (c) Swelling ratio measurements for hydrogels synthesized with varying N,N'-methylenebisacrylamide content.

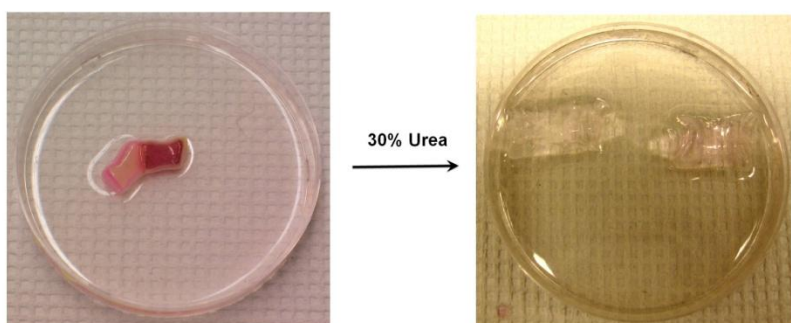


Figure 6.S3. Disruption of hydrogen bonding with urea

Two healed hydrogels (left) separated upon immersion in a 30% (wt/vol) solution of urea (right).

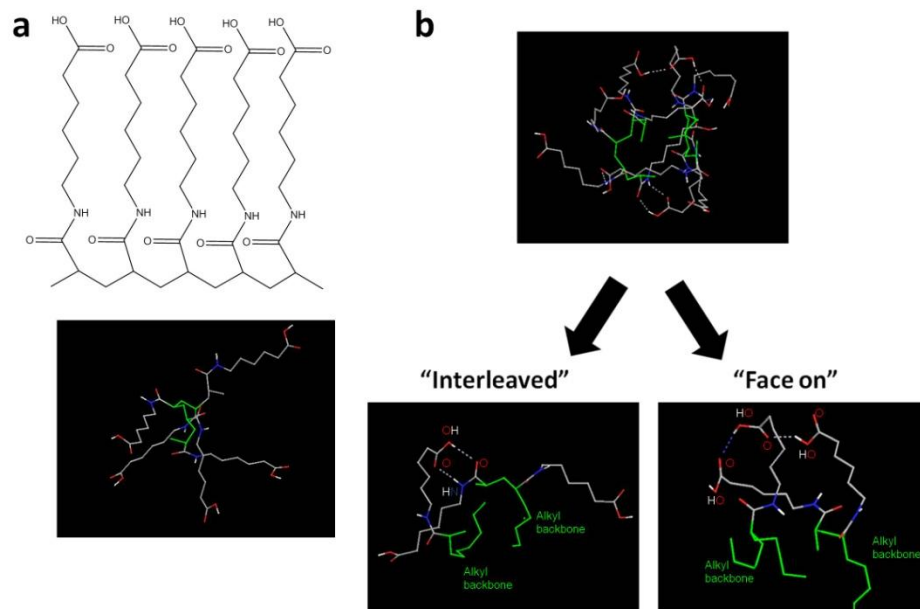


Figure 6.S4. Steric feasibility of deduced H-bonding configurations

(a) Chemical structure of five unit oligomer of A6ACA and its energy-minimized structure (bottom). (b) The energy-minimized structure of two five-unit oligomers facing each other (top) showing both interleaved (bottom, left) and face-on configurations (bottom, right). Dotted lines, intermolecular hydrogen bonds; green, alkyl backbone; gray, CH₂ groups in pendant side chain; blue, nitrogen; red, oxygen; and white, polar hydrogen.

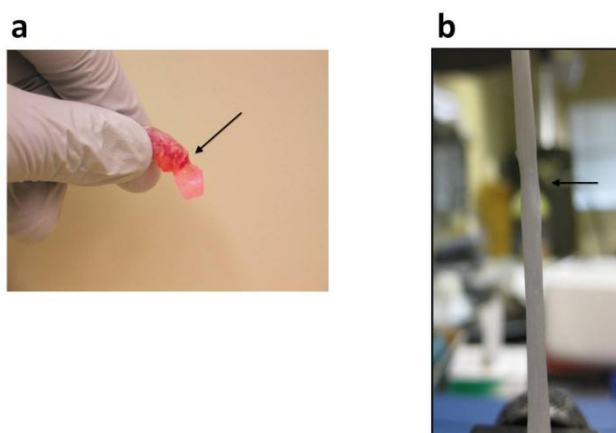


Figure 6.S5. Weld-line interface in the healed hydrogels

(a) Hydrogels healed for 5 min fracture within the bulk region and not at the weld-line.
(b) Hydrogels healed for 24h, which exhibit an opaque color, fracture at the weld-line during tensile testing. The arrows indicate the weld-line.

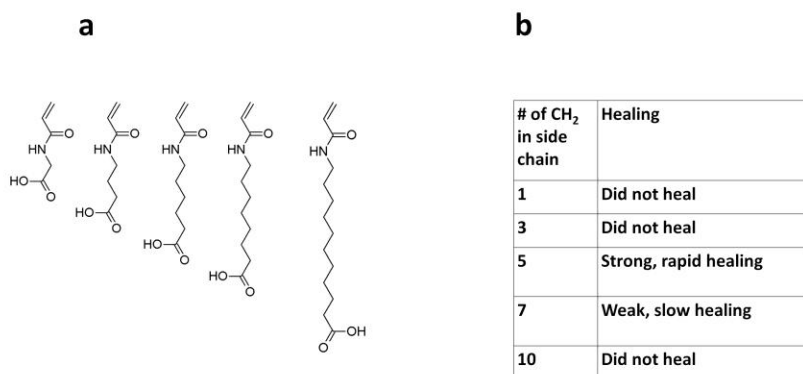


Figure 6.S6. Effect of side-chain length on healing efficiency

(a) Chemical structure of N-acryloyl modified amino acids used to investigate the effect of side-chain length on healing ability (from left to right) N-acryloyl glycine (1 CH₂ group), N-acryloyl 4-aminobutyric acid (3 CH₂ groups), N-acryloyl 6-aminocaproic acid (5 CH₂ groups), N-acryloyl 8-aminocaprylic acid (7 CH₂ groups), and N-acryloyl 11-aminoundecanoic acid (10 CH₂ groups). (b) Table summarizing the effect of pendant side-chain length on healing

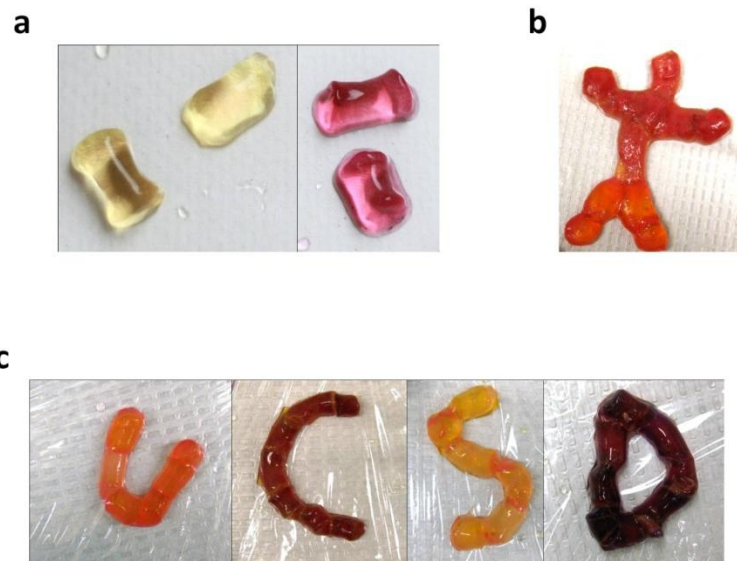


Figure 6.S7. A6ACA hydrogels fused to form complex architectures

(a) Unhealed cylindrical hydrogels, dyed maroon and yellow respectively, and the same hydrogels healed to form a humanoid figure (b) and the letters 'UCSD' (c). Yellow hydrogels were used to assemble the 'U' and then separated by exposure to pH 13. They were then healed to form the 'S'. This procedure was repeated for 'C' and 'D'. The differences in the order at which the four letters were assembled explains the differences in swelling and color.

6.9. Tables

Table 6.1. Assignments of IR and Raman bands

Low pH		High pH		Assignments
Raman	IR	Raman	IR	
-	3,310	-	-	N-H stretch, H bonded (Colthup <i>et al.</i> , 1975; Roeges, 1994)
2,929; 2,866	2,925; 2,857	2,929; 2,866	2,930; 2,860	C-H stretches
-	2,500 (broad)	-	-	Overtone/ combination bands (Colthup <i>et al.</i> , 1975)
1,714	1,704	-	-	C=O stretch of terminal carboxylic acid (Colthup <i>et al.</i> , 1975)
1,642	1,645	1,643	1,642	Amide (Barbucci <i>et al.</i> , 1989; Colthup <i>et al.</i> , 1975; Roeges, 1994)
1,624	1,627	-	-	Amide I (H-bonded to -COOH)
1,568	1,547	1,567	1,551	Amide II (Colthup <i>et al.</i> , 1975); overlap with COO- stretch (Colthup <i>et al.</i> , 1975) for high pH-IR
1,444	1,440	1,444	1,440	CH ₂ bend deformation (Colthup <i>et al.</i> , 1975)
-	-	1,409	1,403	COO- stretch (Barbucci <i>et al.</i> , 1989)
1,355	1,386	-	-	OH deformation (Colthup <i>et al.</i> , 1975)
1,309; 1,265	-	1,309; 1,265	-	CH ₂ wag or twist (Dong <i>et al.</i> , 1997) and Amide II
-	1,271- 1,192	-	-	C-O stretch coupled with C-O-H in-plane bend (Colthup <i>et al.</i> , 1975)
1,169- 1,083	1,163- 1,081	1,170- 1,085	1,164- 1,080	C-CH ₂ and N-CH ₂ stretches (Dong <i>et al.</i> , 1997)
912- 843	900 (broad)	947	860; 989	Unassigned
-	-	561	-	COO- rock

Table 6.2. Composition of buffers with varying pH

Buffer pH	Composition
1	21.17% 0.2 M potassium chloride + 72.83% 0.2 M hydrochloric acid
3	69.15% 0.1 M potassium hydrogen phthalate + 30.85% 0.1 M hydrochloric acid
4	99.8% 0.1 M potassium hydrogen phthalate + 0.02% 0.1 M hydrochloric acid
4.5	85.18% 0.1 M potassium hydrogen phthalate + 14.82% 0.1 M sodium hydroxide
5	68.87% 0.1 M potassium hydrogen phthalate + 31.13% 0.1 M sodium hydroxide

6.10. References

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CHAPTER 7: Future Directions

The work presented in this dissertation demonstrates the synthesis of biomineralized composites that mimic bone, and demonstrate great promise for use as synthetic grafting substitutes through their ability to promote osteogenesis of human mesenchymal stem cells *in vitro*, bone formation in a rat subcutaneous implantation model, and spinal fusion in a rat posterolateral fusion model. These results present a foundation supporting the utility of biomineralized synthetic matrices as biomimetic, osteoinductive synthetic bone grafts; there are however, a number of potential avenues for extending this work, both from a translational perspective, as well as gaining fundamental insights into tissue mineralization. This chapter will discuss some of these potential directions for future exploration.

7.1. Further evaluation of the synthetic grafts for clinical translation

Evaluation of the synthetic grafts in additional healing models is an area that warrants immediate investigation. The work presented in this dissertation demonstrates the utility of these grafts in a posterolateral fusion model as well as in a subcutaneous Urist ectopic bone induction model. The applicability of this material in posterolateral fusion is a highly promising finding, particularly as posterolateral fusion has been noted to be typically more challenging than other *in vivo* models of osteogenesis.. In order to investigate their utility in the broad range of applications for bone grafts, it is important to probe their ability to promote bone formation in a variety of models, such as

tibial/femoral defect models, cranial/calvarial defect models, as well as for other spinal fusion procedures such as interbody fusion. The success of the material in the challenging posterolateral fusion model does however suggest that the material has a high probability of success in the other models.

In addition, it is vital to explore the osteoinductivity of this material across a variety of species, as inter-species variability has previously been noted in material-induced osteoinduction. However, the work presented here demonstrates the osteoinductivity of the material in rats; traditionally, material-induced osteoinduction has been reported to be far more challenging in rodent models than in larger animal models such as pigs, dogs, sheep and primates. Several calcium phosphates have been widely demonstrated to promote bone formation in large animals and not in rodents (Yang *et al.*, 1996; Yuan *et al.*, 2006); however, the reverse (wherein materials promote bone formation in rodents but not in small animals) has not been reported. In other words, the osteoinductive capacity of synthetic materials in rodent models can generally considered a reliable and conservative estimate of their osteoinductive capacity in larger animal models and in humans. As a result, it is possible that the osteoinductivity of the biomineralized A6ACA-PEGDA grafts in larger animal models would be at least comparable to, if not enhanced over the osteoinductivity exhibited by the A6ACA-PEGDA grafts exhibited in rats during this work.

While these grafts present a potential alternative to recombinant growth factors and autografts, one must not ignore the established success of using grafts based on viable bone and growth factors in a clinical setting. Indeed, a combination of the

synthetic grafts presented here with either autografts or recombinant growth factors could provide for a system which achieves comparable clinical efficacy while mitigating some of the risks currently associated with these procedures. For example, the biom mineralized A6ACA-PEGDA grafts could be used as a carrier of osteoinductive recombinant growth factors. Growth factors such as BMPs could be either pre-adsorbed or incorporated into the mineralized phase, due to their high affinity for calcium phosphates (via both direct binding as well as through interaction with mineral-binding proteins such as fetuins) This could allow for a reduction in the required dosage of the recombinant factors, thereby reducing the costs and risks associated with the high doses of these factors currently used. Indeed, calcium phosphate-based materials have been widely used as carriers for recombinant proteins for the purpose of osteogenesis. Moreover, a combined use of these grafts with autografts (i.e. using them as autograft extenders) could allow for reduction in the amount of autologous bone required, thereby reducing the risk of donor site morbidity while still obtaining the excellent clinical outcomes associated with the use of autologous bone.

An interesting observation in the course of this work (see Chapter 4) was that in addition to ectopic bone formation, the presence of a mineralized phase dramatically improved host cell infiltration and vascularization of the synthetic matrices. This is in agreement with other studies, which have demonstrated that inorganic mineralized matrices can not only adsorb pro-angiogenic factors from serum, but also dramatically increase their production in host cells exposed to these materials (Day, 2005; Gorustovich *et al.*, 2010). This suggests that these mineralized materials could not only be utilized in

applications requiring osteoinductivity, but also to improve the vascularization of synthetic matrices; current methods of improving angiogenesis involve use of pro-angiogenic cells, growth factors or peptides (Bouhadir and Mooney, 2001; Licht *et al.*, 2003; Moon *et al.*, 2010). By tailoring the amount of minerals present, it is possible that matrices could be endowed with their pro-angiogenic effect without the accompanying osteoinductivity, expanding potential applications of these mineralized matrices beyond the orthopedic applications explored in this dissertation.

7.2. Creating degradable synthetic osteoinductive grafts

The grafts investigated in this dissertation are highly promising due to their excellent osteoinductivity and biocompatibility but in their current form, lack biodegradability. Although PEGDA hydrogels such as the one presented in this work contain ester bonds which can be broken through acid or base hydrolysis, it is generally regarded as highly stable and for all intents and purposes, practically non-degradable *in vivo* due to the lack of such extreme pH conditions. Engineering synthetic grafts that exhibit osteoinductivity similar to the biomineralized A6ACA-PEGDA grafts, yet would be capable of degradation *in vivo* would allow for the gradual replacement of the graft material with neo-bone tissue, and allow for its effective remodeling into mature bone. This can be achieved by replacing the PEGDA network presented here with a network containing moieties that can be cleaved under physiological conditions, while still retaining the osteoinductivity and biocompatibility endowed by the presence of A6ACA and biomineralization. These moieties could be cleaved via a number of mechanisms such as hydrolysis (Martens *et al.*, 2003), enzymatic degradation (He and Jabbari, 2007)

and photocleavage (Kloxin *et al.*, 2009). Among these mechanisms, enzymatic degradation and photocleaving are expected to be significantly impeded due to the presence of the mineralized layer which is likely to reduce accessibility of the respective degradative agents to the underlying polymeric substrate. Along with the specific mode of degradation, the degradation products are an important consideration; it is vital that the degradation products be non-toxic and not trigger unfavorable inflammatory responses. While poly (l-lactide-co-glycolic acid) (PLGA)-based and poly (L-lactic acid) (PLLA-based) materials have been widely used for their degradable nature, the acidic nature of their degradation products has been reported to cause inflammation. However, as the dissolution of apatites tends to lead to a slight shift in pH towards alkalinity (Linhart *et al.*, 2001), it is possible that mineralized composites could compensate for the acidic nature of the degradation products in this manner. In fact, a previous study illustrated that PLGA/calcium phosphate composites promoted improved cell viability when compared to non-degradable mineralized matrices, possibly due to the fact that acidic degradation products from the PLGA and the alkaline shift from the mineral dissolution compensate each other (Linhart *et al.*, 2001).

The rate of degradation is a critical factor for the design of synthetic grafts; even in non-load-bearing applications, an excessively high degradation rate could lead to a premature loss of the matrix-based instructive cues required to stimulate *de novo* bone formation by the recruited endogenous host progenitors, while an excessively slow degradation rate would impede the *de novo* bone formation as well as the subsequent remodeling of bone (Alsberg *et al.*, 2003). A promising strategy for the development of

synthetic bone grafts capable of degradation involves mimicking the native processes involved in bone resorption *in vivo*. During bone resorption, osteoclasts first create an acidic microenvironment at the desired site of resorption; this leads to the dissolution of the mineralized phase. The osteoclasts then subsequently secrete a number of proteases such as cathepsins K and O2 which subsequently degrade the osteoid (Bossard *et al.*, 1996; Brömme *et al.*, 1996). Capitalizing this process, biomineralized bone grafts wherein the polymeric phase contains moieties cleavable specifically by these proteases can not only mimic native bone by way of composition, but also by their ability to undergo osteoclast-mediated resorption/degradation. Previous studies have illustrated that synthetic materials containing protease-specific peptide moieties can then undergo degradation through the action of these proteases (Patterson and Hubbell, 2010). Such osteoclast responsive materials allow for the ‘smart’ degradation of the materials only at the time of bone remodeling- the optimal time for their degradation, thereby mitigating the need to design materials capable of resorbing at a pre-determined optimal degradation rate. This is particularly important as different patients would likely have require grafts with different rates of degradation due to patient-to-patient variation in intrinsic osteogenic capacity. This also mitigates the issue of the presence of the mineralized phase, which could serve as an impediment to the degradative agents as mentioned previously. Indeed, such materials that display osteoclast-specific degradation have already been recently reported (Hsu *et al.*, 2011)) and thus, present a promising potential option for degradable synthetic bone grafts.

7.3. Cellular and molecular mechanisms underlying the osteoinductivity of calcium phosphates

Another important extension of this work is to investigate the mechanisms underlying the osteoinductivity of these mineralized composites, especially as this mechanism has been suggested to differ significantly from the osteogenic pathways triggered by soluble cues such as BMPs and osteogenic media (Lin *et al.*, 2009). The role of dissolution-reprecipitation and the resultant increased local Ca^{2+} and PO_4^{3-} ion concentrations in particular often been invoked by numerous studies over the past decade; however, the cellular mechanisms underlying this effect have only recently been investigated. Barradas *et al.* recently reported a cellular mechanism through which Ca^{2+} ions promote osteogenic differentiation of hMSCs. They found that L-type voltage gated calcium channels were chiefly responsible for the osteogenic differentiation, through a c-Fos-mediated pathway (Barradas *et al.*, 2012). Somewhat counter-intuitively, calcium-sensing receptor (CaSR) was found to not play a role in Ca^{2+} -mediated osteogenic differentiation despite its demonstrated effect in skeletal maintenance and development as illustrated in the literature (Chang *et al.*, 2008); it is important to note however, that CaSR-independent calcium sensing has indeed been demonstrated in osteoblasts (Pi *et al.*, 2000; Pi *et al.*, 1999).

Recent work from our lab has established the role of a sodium-phosphate cotransporter SLC20A1 and downstream adenosine signaling during the *in vitro* osteogenic differentiation of hMSCs mediated by the role of soluble phosphate released from biomineralized synthetic matrices (Shih *et al.*, 2012). SLC20A1 has previously been

implicated in the expression of osteogenic phenotype in vascular smooth muscle cells during vascular calcification in hyperphosphatemic patients, thus making it a promising candidate for phosphate-induced osteogenesis in hMSCs (Li *et al.*, 2006). This study revealed that both, hMSCs cultured on mineralized A6ACA hydrogels in the absence of exogenous soluble osteogenic stimuli, as well as hMSCs cultured in media containing increased phosphate concentrations exhibited increased expression of SLC20A1. The siRNA-mediated knockdown of SLC20A1 was found to reduce the expression of osteogenic markers on mineralized hydrogels as well as in high-phosphate culture; this study demonstrated a mechanism through which the phosphate ions provided by the mineralized substrate are internalized by hMSCs via SLC20A1 and then used for mitochondrial ATP synthesis. This ATP is then secreted by the cells and degraded to adenosine, which then triggers an intracellular osteogenic cascade via binding to A2B adenosine receptors. The role of adenosine signaling and specifically, A2B adenosine receptors on osteogenesis and bone homeostasis *in vivo* has recently been reported in as well (Carroll *et al.*, 2012; Evans and Ham, 2012; Evans and Ham, 2007) , suggesting that this signaling pathway may hold great promise in exploring the cellular response to biomineralized matrices.

An additional mechanism that has been stated as underlying the osteoinductive capacity of specific calcium phosphates is the selective binding of growth factors. Numerous studies have suggested that calcium phosphate-based materials could adsorb pro-osteogenic growth factors such as BMPs from body fluid, leading to their concentration in the implant and subsequently allowing for bone formation (Lee *et al.*,

2011; Urist *et al.*, 1984). While previous work does indeed suggest that BMPs are able to directly bind to calcium phosphates, other studies also suggest that this binding could be through the binding of calcium phosphates to fetuin and the subsequent binding of fetuin to BMPs (Binkert *et al.*, 1999; Price and Lim, 2003). As this mechanism could harness the body's endogenous store of osteoinductive growth factors, this certainly merits further investigation and experimental validation.

7.4. Effects of biomineralized matrices on other cell types *in vitro*

The work here demonstrates the ability of these mineralized composites to direct the osteogenic differentiation of mesenchymal multipotent progenitors. However, the effects of such matrices on other cell types could also produce some interesting insights on mineralization processes *in vivo*, particularly those relating to pathological calcification. The response of aortic smooth muscle cells to these biomineralized matrices could provide insights into the role of substrate-induced cues during dystrophic calcification in vasculature. This is typically observed in patients with end stage renal disease and patients with severe hypercalcemia and/or hyperphosphatasia (Moe and Chen, 2004), and is characterized by apatite deposition in soft vascular tissue. While this was previously thought to be due to passive precipitation of calcium phosphates, recent work has demonstrated that vascular calcification is characterized by the transition of vascular smooth muscle cells to an osteoblast-like phenotype, accompanied by the expression of bone specific extracellular matrix markers and the deposition of calcified matrix (Abedin *et al.*, 2004; Vattikuti and Towler, 2004). More recent work has also demonstrated that calcium phosphate deposition is critical to this phenotypic transition

(Villa-Bellosta *et al.*, 2011); however, the effect of substrate-based cues from the calcified matrix on this phenotypic transition has not been explored. In other words, a critical question remains: is this phenotypic transition (and subsequent calcified tissue formation) triggered by an initial passive deposition of calcium phosphate due to supersaturation, or do the supersaturated concentrations trigger the transition as soluble cues and then subsequently lead to the osteogenic phenotype?

The biom mineralized A6ACA composites could thus be used to gain insights into these substrate-based cues and address this issue; culturing vascular smooth muscle cells on these materials without the addition of calcification-inducing medium supplements (such as increased phosphate ions) to the medium would provide insights regarding the role of pre-calcified matrix on the phenotypic transition. Calcified hydrogels thus represent a promising *in vitro* platform for investigation into this phenomenon, as well as for evaluation of potential therapies. In addition to vascular calcification, these materials could also be utilized to gain insights into heterotypic intramuscular bone formation in conditions such as myositis ossificans progressiva, traumatic myositis ossificans, as well as heterotypic bone formation observed during arthroplasty and surprisingly, in some patients suffering from traumatic brain injuries and neurological disorders (Pape *et al.*, 2004).

In addition to the calcium phosphate matrices explored here, matrices could also be synthesized to gain insights into the deposition of other calcium salts in physiological processes. For example, biom mineralized matrices that can promote the growth of calcium carbonate could be used to mimic the exceptional strength endowed to shells of marine

invertebrates via biomineralization (Naka and Chujo, 2001). Furthermore, the development of matrices mimicking calcium oxalate could yield platforms studying the deposition of these minerals during the formation of kidney stones; the formation of calcium oxalates has also been demonstrated to be driven by acidic amino acid residues, which could provide insight into their formation *in vivo* (Wang *et al.*, 2006). These are only a few of the examples of potential applications of biomineralized matrices incorporating calcium-based minerals.

7.5. Conclusions

In summary, the mineralized composites described in this work hold great promise as both, a synthetic system for bone regeneration in translational applications as well as a platform for investigating substrate based cues arising from biomineralized matrices and their effect on various physiological processes *in vitro*. Specifically, areas of future development include imbuing the grafts with degradability, elucidation of mechanisms underlying the osteoinductivity of these matrices described in this work as well as exploring their utility to obtain insights into dystrophic and pathological calcification.

7.6. References

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