Volume 7, Issue 1, 2017, 1989 - 1994 ISSN 2069-5837

Biointerface Research in Applied Chemistry

www.BiointerfaceResearch.com

Review Article Open Access Journal

Received: 13.12.2016 / Revised: 28.01.2017 / Accepted: 12.02.2017 / Published on-line: 15.02.2017

Utility of amorphous calcium phosphate-based scaffolds in dental/biomedical applications

Diane R. Bienek [1, *], Drago Skrtic [1]

[1] ADA Foundation, Volpe Research Center, Gaithersburg, MD 20899

*corresponding author e-mail address: ***bienekd@ada.org***

ABSTRACT

Calcium phosphate (CaP) materials are important inorganic constituents in biological hard tissue. CaPs, including amorphous calcium phosphate (ACP) have been widely applied in dental and biomedical applications, such as tissue engineering. Scaffold constructs are commonly used as templates to create a biomimetic environment. This review considers ACP scaffold fabrication techniques, including tissue-engineered constructs with intrinsic incorporation of ACP as well as scaffolds formed via precipitation of mineralized solutions on a substrate. Attention is given to the approaches used to assess cellular and molecular responses elicited by ACP scaffolds, such as biocompatibility, cell conductivity, cell adhesion, cell differentiation, phenotypic profiles, and gene expression. Bioactivity of composite ACP scaffolds can be enhanced by incorporating biomolecules to create multi-functional properties. Herein we summarize the use of antibiotics, growth factors, and gene delivery systems to create multi-functional ACP scaffolds. Inasmuch as CaP materials have been investigated as drug delivery systems for many years, we briefly consider the potential of integrating these systems with existing ACP scaffold constructs and the potential for precision medicine.

Keywords: *regenerative medicine, tissue engineering, amorphous calcium phosphate, scaffold.*

1. INTRODUCTION

The field of regenerative medicine is a branch of tissue engineering (TE) that considers the process of replacing, regenerating cells, tissues or organs, to restore or establish normal function [1]. Scaffold constructs are commonly used to create an adequate biomimetic environment by providing mechanical /structural support to facilitate optimal cell growth and function. For the development of novel TE scaffolds, the following properties warrant consideration: 1) ability to deliver cells; 2) cellular conductivity; 3) biodegradability; 4) mechanical properties; 5) porosity; 6) pore sizes; 7) irregular shape fabrication ability; and 8) potential for commercialization (summarized by Chen *et al*., (2006) [2]). To achieve the desired scaffold qualities, biomaterials are often a hybrid of organic-inorganic components.

Natural and synthetic polymers are frequently used for fabrication of composite scaffolds. Natural polymers, such as proteins, polysaccharides or polynucleotides [3], typically offer an affable interface for cells. Synthetic polymers offer a possibility of tailoring their physicochemical and mechanical properties for specific applications. Frequently used synthetic polymers/ copolymers are polylactic acid (PLA), poly-L-lactic acid (PLLA), poly-D, L-lactic acid (PDLLA), poly lactic-co-glycolic acid (PLGA), poly(methyl methacrylate) (PMMA), and polycapro-lactone (PCL).

Compounds from the calcium phosphate (CaP) family have been widely applied in the biomedical field. Various properties of amorphous calcium phosphate (ACP), the only non-crystalline member of the CaP family, render it a promising biomaterial for tissue repair and regeneration. For example, ACP supports regeneration of hard tissues, which involves the synthesis and extracellular assembly of collagen I matrix framework of fibrils followed by its mineralization (reviewed by [4]). Secondly, the transformation of ACP precursors into thermodynamically stable apatite is involved in the remineralization of collagen fibrils [5, 6]. Thirdly, ACP scaffolds have excellent bioactivity supporting cell adhesion [7-12], growth [8, 9, 11, 13], and differentiation [7, 10, 14, 15]. Further, a biomimetic apatite layer has potential to serve as a vehicle for the release of biomolecules, such as growth factors, enzymes, or drugs [16].

Hybrid ACP and polymeric biomaterial TE constructs can assume a variety of architectures, depending on the fabrication technique. For orthopedic applications, scaffolds containing ACP have been designed for repair, restoration or regeneration of bone [2, 7, 10-14, 17-26], ligaments/tendons [27], and cartilage [28]. In dental applications, ACP scaffolds have been developed for remineralization of dentin [6, 29].

The objective of this review is to focus on ACP scaffold fabrication techniques and approaches used to assess cellular responses to these TE constructs. As biomedical engineering applications advance, there is an increasing interest to create multi-functional scaffolds. Accordingly, this review also considers the release/absorption of biofactors from ACP scaffolds and the potential for precision medicine.

2. SCAFFOLD FABRICATION

2.1. Intrinsic Incorporation of ACP into Scaffolds. Scaffold architectures for TE applications include a variety of forms (i.e., nanofibers, foams, amorphous particles, etc.). As cell behavior can be influenced by the scaffold's physical properties, the appropriate fabrication technique needs to be carefully considered for the intended application.

2.1.1. Nanofiber Scaffolds. To achieve electrospun scaffolds with better mechanical stability and bioactivity, ACP has been integrated with synthetic polymers [7, 11, 14, 21, 27]. For bone

TE applications, electrospun ACP/PLGA scaffolds have been successfully seeded with mesenchymal stem cells [11] or human adipose-derived stem cells (ASC) [7, 14, 21]. To overcome problems in critical size bone TE, some studies have developed three-dimensional conformations with ACP/PLGA scaffolds. For example, Gao *et al*., (2014) used tubes prepared with electrospun mesh to co-culture pre-differentiated endothelial cells and osteoblasts. Using this system, they reported that the cell-cell interactions seem to drive the differentiation of both cell types, while slightly reducing the proliferation rate [21]. Baumgartner and colleagues (2015), produced a critical size bone-biomimetic construct seeded with human ASC's by stacking eight disks of ACP/PLGA electrospun constructs [14].

While electrospinning has multiple applications and is valuable to study cellular interactions, the *in situ* deposition of nanofibers is limited because of safety requirements and technical limitations. Blow spinning (also known as airbrushing) is a technique that is gaining attention in TE, as the resultant nanofibrous structures can be used to create a biomimetic environment to allow on-demand fabrication of conformational nanofiber mats for precise and site-specific construction [30]. Using zirconium-modified ACP (Zr-ACP) dispersed in biocompatible polymers, penetration of human bone marrow stromal cells (hBMSC) within the airbrushed scaffolds exceeded more than twice the cell penetration within conventional electrospun networks [22]. Long-term cultivation of hBMSCs on airbrushed composite fiber scaffolds with different Zr-ACP loads (0%, 5%, or 20% (w/w)) demonstrated similar DNA content patterns. Further analyses revealed that, within a given Zr-ACP concentration, cellular DNA levels can vary greatly and depend on the polymer type. For example, hBMSCs incubated (1 or 16 days) with 20% Zr-ACP PCL scaffolds yielded significantly ($P < 0.05$) higher DNA levels than those cultivated with 20% Zr-ACP dispersed in PDLLA [18].

2.1.2. Thermally-induced Phase Separation. Thermally-induced phase separation is a common method in scaffold design that takes advantage of the thermodynamic instability of polymer solutions at particular temperatures. In general, it is a multi-step process involving polymer dissolution, phase separation, solvent extraction, freezing, and freeze-drying. Thermally-induced phase separation was used to prepare a highly porous ACP/PLLA composite. Through a series of experiments, it was demonstrated that: 1) ACP aggregates covering the PLLA pore walls could undergo a rapid phase transformation and morphologic variation into flake-like crystallites when soaked in PBS [10]; 2) ACP/PLLA composite enhanced osteoblastic adhesion and differentiation [10]; 3) basic fibroblast growth factor (bFGF) could be adsorbed and immobilized on the ACP/PLLA scaffold and released in a sustained manner after an initial burst [28]; and 4) ACP/PLLA incorporated with bFGF could successfully resurface an osteochondral defect and restore subchondral bone in a rabbit model [28]. Additionally, it was demonstrated that the ACP/PLLA composite promote protein adsorption and osteoblastic adhesion, which may be regulated by the integrin-focal adhesion kinase pathway [12]. The thermal phase separation technique was used to fabricate PLGA/Zr-ACP composites [31]. An advantage of using Zr-hybridized, pyrophosphate-stabilized ACP includes retarding the dissolution of ACP and subsequent conversion to hydroxyapatite (HA) in aqueous environments. Further, this hybridized form allows controlled release of calcium (Ca) and phosphate (P) ions, as well as providing better mechanical stability to the polymer scaffold.

2.1.3. Salt-leached Gradient. Chatterjee *et al.,* (2011) applied a spray drying technique for the preparation of nano-ACP (nACP) particles [8]. The resulting nACP particles were then combined with PCL using a salt-leached gradient scaffold approach to fabricate 3D porous constructs. As gradients were suitable for measuring effects of scaffold composition on osteoblast adhesion at short time periods (1 day), they indicated that this approach could be used as a high-throughput method for screening cell-biomaterial interactions.

2.2. Scaffold Formed via Solution Precipitate. For the development of TE constructs, various substrates have been used to elicit nucleation and CaP crystal growth. For TE applications, we herein focus on the scaffold substrate and approach to form crystals.

2.2.1. Bioactive Glass. Surface reactive glass ceramics are regarded as favorable biomaterials in TE because of controllable degradability and the ability to promote cell adhesion, growth, and differentiation. Using a ceramic-coated sacrificial polymer foam template and optimal sintering conditions, a highly porous 45S5 Bioglass-derived glass-ceramic scaffold was fabricated [2]. A unique feature of this scaffold is that the crystalline phase that provides the scaffold mechanical competence, can transform into an ACP phase after 28 days immersion in simulated body fluid (SBF). An *in vitro* study using MG63 osteoblast-like cells indicated that 45S5 Bioglass-derived glass-ceramic scaffolds have excellent cell infiltration and proliferation at the central regions of the scaffold [9]. Further, the mechanical stability of this scaffold was maintained while the scaffolds biodegraded in cell culture medium. More recently, the gel-cast foaming technique was adapted to fabricate melt-derived bioactive glass scaffolds [25]. This report indicated that ACP could be formed on bioactive glass scaffolds in a shorter time period (i.e., after 8 h of immersion in SBF and crystallized hydroxycarbonate apatite formed within 3 days). Furthermore, the resulting porosity and compressive strength of these bioactive glass scaffolds are expected to render them as a suitable network for vascularized bone regeneration.

2.2.2. Nanogold Membranes. To make a malleable TE construct that could be molded for bone-implant applications, Rautaray and Sastry (2005) synthesized free-standing nanogold membranes in a polymeric background for growth of inorganic crystals [32]. They demonstrated that gold nanoparticles in the membrane can be functionalized with amino acids (aspartic acid and cysteine) and a bifunctional molecule. The type of CaP formed (ACP or HA) was controlled by incorporation of nanogold surface modifier,

2.2.3. Hydrogels. Hydrogels are suitable for TE constructs due to their highly swollen network structure, ability to encapsulate cells and biomolecules, and efficient mass transfer [33]. ACP (or ACP precursors) have been combined with natural polymers, such as

gelatin [17], collagen [13], or chitosan [23], to produce hydrogel scaffolds with similarities to bone matrix.

To resemble the composition and structure of natural bone from the nano- to macro-scale, Hu *et al.,* (2016) fabricated hierarchically biomimetic collagen-apatite scaffolds with both intrafibrillar and extrafibrillar mineralization [13]. Using a one-step bottom-up approach, intrafibrillar and extrafibrillar mineralized collagen apatite scaffolds were fabricated using polyacrylic acid and modified SBF solution to form stabilized ACP nanoprecursors. In this system, sodium tripolyphosphate was used as a templating analog to regulate deposition of apatite within the collagen fibrils. The resulting collagen-ACP scaffold resembled natural bone (regarding composition and structure) and demonstrated biocompatibility with a pre-osteoblastic cell line.

A double diffusion method was used to prepare a gelatin/ACP nanocomposite scaffold at 4°C [17]. To predict the behavior of this scaffold in the human body, these scaffolds were incubated in SBF solution. After a 48 h incubation at 37°C, the mineral phase was transformed into nanocrystalline HA. Altogether, this study concluded that if this gelatin/ACP nanocomposite scaffold were implanted into the body, it would be converted to HA crystals through a process similar to natural bone formation.

For dental applications, nanocomplexes of carboxymethyl chitosan/ACP gel scaffolds were developed to facilitate remineralization of dentin. Briefly, using a single layer collagen model and a model of deep caries, ACP nanoparticles were released from scaffolds of carboxymethyl chitosan/ACP nanocomplexes and then infiltrate into collagen fibrils via gap zones to accomplish intrafibrillar mineralization of collagen [29].

2.2.4. Starch/PCL-based. As a rapid prototyping technology, starch/PCL-based scaffolds were produced using a 3D plotting technology [16]. The nucleation and subsequent growth of apatite layers on this scaffold surface and in the interior was studied under static and dynamic mineralizing conditions (i.e., immersion in SBF). These authors reported that dynamic conditions can accelerate the formation of apatite in a pre-established apatite layer while maintaining composition, crystallinity, and chemical structure.

3. CELL/TISSUE FUNCTION

Initial biological evaluation of CaP scaffolds is commonly assessed *in vitro* using stem cells [7, 11, 14, 15, 18, 21, 22] osteoblast-like cells [8, 10, 13, 26, 27, 34], and epithelial-like cells [35]. In most cases, the cytotoxic potential of the scaffolds is evaluated using a static system that renders direct contact between the cell and the scaffold. Inferences of cytocompatibility of ACP scaffolds are commonly drawn from cellular morphological observations, metabolic activity (i.e., tetrazolium reduction assays), total protein, or DNA content.

For ACP scaffolds, many reports have demonstrated excellent bioactivity; however, the associated cellular and molecular processes are not fully understood. Adipogenic, chondrogenic, and osteogenic differentiation of ASC cells on ACP scaffolds have been assessed using histologic stains [7, 14]. Using a perfusion bioreactor, Baumgartner *et al*., (2015) demonstrated that the onset of osteogenesis (<1%) was triggered by dynamic culture conditions; however, endothelial cell differentiation and chondrogenesis was not triggered. They proposed that the resulting biodegradable biominerizable critical size nanocomposite with human ASCs in a gradient manner could offer a starting point to achieve lineage specific differentiation using medium supplementation [14]. Osteogenic differentiation has also been assessed by examining levels of alkaline phosphatase activity as an osteoblast marker. Briefly, human osteoblastic MG63 cell cultures showed that the ACP/PLLA composite scaffolds had better cell adhesion and alkaline phosphatase activity than PLLA alone [10].

Phenotypic profiles of ASC seeded onto electrospun nanocomposite PLGA/ACP material were characterized by flow cytometry [7]. Typical stem cell markers (CD13, CD29, CD44, and CD105) were highly expressed. Whereas markers for endothelial cell lineage/differentiation (CD31, CD34, or CD146) had no/low expression.

To gain insight into the mechanisms, some researchers have used reverse transcription polymerase chain reaction (RT-PCR) to evaluate gene expression. Syed-Picard *et al*., (2013), conducted a series of experiments using a scaffoldless 3D dental pulp cell construct to better understand the osteoinductive properties of ACP. They demonstrated that ACP alters cellular functions and tissue differentiation patterns through release of Ca ions, which modulates connexin 43-mediated gap junctions [36]. Cell adhesion to composite scaffolds fabricated by the thermally-induced phase-separation technique was assessed with human osteoblastic MG63 cells. RT-PCR indicated that the enhanced osteoblastic adhesion to porous ACP/PLLA composite scaffolds may be mediated by the binding of integrin subunits (α1, αv, and β1) and subsequently may be regulated through the focal adhesion kinase signal transduction pathways [12].

Osteochondral repair in rabbits with implanted bFGF and ACP/PLLA hybrid materials (fabricated by the thermally-induced phase-separation technique) exhibited high levels of collagen type II and aggrecan gene expression [28]. An abundance of collagen type I gene message suggested that repaired tissues were immature. Moreover, the detection of collagen type X with implants fabricated with ACP indicated the enchondral ossification process of newly formed cartilage.

TE cellular constructs subcutaneously implanted into mice have been used to analyze the role of ACP in the biomineralization process and its osteoinductive properties. For example, hierarchical levels of early stage mineralization were mapped to increase the understanding of the nanoscale organization of the mineral and organic matrices and the organic-mineral transition [19]. Cedola *et al*., (2014) conducted 3D visualization of engineered bone and soft connective tissue for development of a biomineralization model and to define the role of the collagen matrix and ACP in the organic-mineral transition [37]. Further, the

mechanisms behind the osteoinductive properties of ACP were investigated by examining the effects on cell-cell communication via connexin 43-mediated gap junctions [36].

To assert biocompatibility, local and/or systemic responses in a living system (or tissue) need to be considered. Regrettably, there is a paucity of information regarding the use of ACP hybrid scaffolds *in vivo*, as a TE approach to achieve functional restoration. Rabbit models with surgically-induced defects have been used to evaluate efficacy of ACP hybrid scaffolds to enhance regeneration of injured tissue. Treatment ACP/PLLA scaffold combined with bFGF acted favorably for repair of articular cartilage defects resulting in well-established layer of cartilage, abundance of accumulated cartilaginous extracellular matrix, and high levels of type II collagen [28]. Further, *in vivo* investigation of subchondral bone defects in rabbits indicated that growth factor-loaded CaP-PLA coated tantalum (Ta) porous scaffolds are biocompatible and can provide physical support, structural guidance, and an interface for deposition of extracellular bone-like matrix [26].

4. ACP SCAFFOLDS FOR RELEASE OF BIOFACTORS

Bulk biocompatible materials in composite ACP biomedical scaffolds are known to sustain cell growth, but their bioactivity can be further enhanced by incorporating biofactors to create multi-functional properties.

4.1. Antibiotics. Nardecchia *et al*., (2012) described a co-precipitation technique of ACP and ciprofloxacin crystals during the formation of chitosan hydrogels [23]. In this system, ACP exerted control on the kinetic release of ciprofloxacin from the scaffolds, suggesting that these scaffolds are suitable substrates for delivery of antibiotics.

4.2. Proteins/Growth Factors. Bovine serum albumin was used as a model protein to investigate the release properties of Ta plates coated with CaP-PLA composite [26]. The initial rapid release of bovine serum albumin was associated with the structural and morphological changes occurring during phase transformation from ACP nanospheres to crystalline HA nanosheets, and changes in hydrophilicity as a result of surface morphology alterations.

In addition to release kinetics, it is necessary to consider the stability and degradation rates of exogenous factors. To overcome rapid degradation that can occur when bFGF is injected in a soluble form to the injury site, Huang *et al*., (2007) utilized ACP/PLLA as a carrier. *In vitro* release kinetics showed that at the first day, an initial burst (approx. 31%) of bFGF from the scaffold. Thereafter, the growth factor was released in a sustained manner [28]. Effective delivery of exogenous growth factors *in vivo* was also demonstrated by implanting vascular endothelial growth factor/transforming growth factor beta containing CaP-PLA coated porous Ta scaffold into a subchondral bone defect model. After 12 weeks, deposition of extracellular bone like matrix on the surface of the scaffolds and new bone tissue growth inside the defect and porous scaffold was observed [26].

4.3. Gene Delivery. A surface-mediated gene delivery system was fabricated as an assembly consisting of DNA-ACP nanocomposite spheres on an oxygen plasma-treated polystyrene substrate [35]. Using a luciferase reporter gene assay to assess efficacy of gene delivery, it was demonstrated that controlling co-precipitation parameters (i.e., precipitation time and solution Ca and P concentrations) is important for designing cell-stimulating and biocompatible scaffold surfaces.

5. FUTURISTIC POTENTIAL OF ACP SCAFFOLDS

For over 20 years, CaPs have been investigated as drug delivery systems. CaP materials are deemed excellent drug carriers due to high biocompatibility, large surface area, high drug-loading capacity, tunable degradation, and adjustable particle size/ morphological forms (reviewed by [38]).

Recent reports of ACP for drug delivery have sought to develop approaches where drug release is controlled by stimuli from the physiological environment, such as pH conditions. For instance, ACP vesicle-like nanospheres, using the adenosine 5'-triphosphate disodium salt as a biocompatible phosphorus source, were used as a drug nanocarrier that exhibits a pH-responsive delivery of an anti-cancer drug [39]. Using a microwave-assisted hydrothermal method, ACP porous hollow microspheres were synthesized using soybean lecithin [40] or a block co-polymer (methoxyl poly(ethylene glycol)-block-poly (D, L-lactide) [41] as a template. These studies demonstrated that ACP porous hollow microspheres have a high drug loading capacity, favorable pH-responsive release of docetaxel, and subsequent damage to tumor cells. Lu *et al.,* (2014) developed a magnetic, pH-responsive drug delivery system based on magnetic iron oxide@ACP core-shell hollow microspheres. In medium with a pH of 7.4, drug release was slow, but was significantly enhanced in medium with pH 4.5 due to dissolution of the ACP layer [42].

With advancing technologies, approaches are being developed where drug release can be controlled directly or triggered by an operator with a remote device affecting the injected or implanted drug delivery system [43]. Reviews (not specific to CaP) have summarized technologies for remotely-triggered drug delivery systems that respond to light [43-45], ultrasound [43, 46], magnetism [43, 45, 46], or electrochemical processes [43].

Integration of CaP materials as a drug carrier, triggered drug-delivery systems, and abovementioned ACP scaffold technologies hold the potential to aid tissue regeneration and enhance therapeutic effectiveness to match the individual clinical need.

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7. ACKNOWLEDGEMENTS

This work was supported by the ADA Foundation and, in part, by the National Institute of Dental and Craniofacial Research (grant DE26122).

8. LIST OF ACRONYMS

ACP = amorphous calcium phosphate
ADA = American Dental Association
ASC = adipose derived stem cells
bFGF = basic fibroblast growth factor
Ca = calcium
CaP = calcium phosphate
CD = cluster of differentiation
HA = hydroxyapatite
hBMSC = human bone marrow stromal cell
nACP = nano amorphous calcium phosphate
P = phosphate
PCL = polycaprolactone
PDLLA = poly-D,L-lactic acid
pH = -log (molar concentration of hydrogen ions)
PLA = polylactic acid
PLGA = poly lactic-co-glycolic acid
PLLA = poly-L-lactic acid
PMMA = poly(methyl methacrylate)
RT-PCR = reverse transcription polymerase chain reaction
SBF = simulated body fluid
Ta = tantalum
TE = tissue engineering
Zr-ACP = zirconia-modified ACP