Effect of starch on the mechanical and *in vitro* properties of collagen-hydroxyapatite sponges for applications in dentistry

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Abstract

This study sought to improve the mechanical and blood-absorbing properties of collagen sponges, while keeping them compressible, by incorporating blended hydroxyapatite (HA)-starch. Results were compared with CollaPlug® (pure collagen). The elastic modulus increased from 1.5 ± 0.2 kPa for CollaPlug® to 49 ± 8 kPa for sponges of composition 1:4:10 (collagen:HA:starch, by weight). The modified microstructure and surface area provided by the starch granules on the sponges improved cell **viability**. Sponges with composition 1:4:10 maintained their blood-clotting capability with almost no change from 5 to 15 min after contact with blood, while CollaPlug® diminished to about half its capacity to absorb blood and form clots. Incorporation of HA-starch into the sponges with composition of 1:4:10, increased the elastic modulus of the collagen-HA sponges, making them more structurally robust. The **viability** of cells and the blood-clotting capability increased with starch incorporation.

Keywords: *Starch, collagen, sponge, dental filler, blood-clotting, CollaPlug®*

1. Introduction

Spongy scaffolds have several applications in dentistry, one of them as dental fillers (Araujo-Pires et al., 2015; Go, Teh, Tan, Zhang & Teo, 2015). After tooth extraction, reduction of the alveolar ridge occurs as a natural physiological response during socket healing process (Hansson & Halldin, 2012; Leblebicioglu et al., 2013). However, preservation of the alveola is necessary when placement of a dental implant is chosen as next step (Araujo-Pires et al., 2015; Go et al., 2015; Wang & Lang, 2012). When dental fillers or dental plugs are used after tooth extraction, vertical and horizontal dimensions of the alveolar ridge are significantly less contracted (Thalmair, Fickl, Schneider, Hinze & Wachtel, 2013; Vignoletti et al., 2012). Sponges are also used as internal matrices to act as barriers to prevent overextension of endodontic sealers (Zou, Liu, Yin, Li & Xie, 2008), which in turn may result in injury of the inferior alveolar nerve, for example (López-López, Estrugo-Devesa, Jané-Salas & Segura-Egea, 2012). Materials for such applications should be easy to process and be capable to stop bleeding or hemorrhage by promoting blood clotting (hemostasis) (Chang, Huang, Yang, Kuo & Lee, 2012; Soares et al., 2015; Zou et al., 2008). Thus, sponges are considered optimal not only to maintain the dental cavity after tooth extraction (Kim, Yun, Lee, Ahn & Kim, 2011; Serino, Biancu, Iezzi & Piatelli, 2003) by reducing alveolar bone resorption (Serino et al., 2003), but also as an internal matrix when placed through furcation perforations (Zou et al., 2008). Considering these characteristics, collagen sponges have been used as wound dressings for dental applications (Ohno et al., 2004; Taira, Sasaki, Saitoh, Nezu & Araki, 2006), among others.

A sponge made of collagen is inherently biocompatible, and is also highly porous, a requirement to allow cell infiltration and exchange of oxygen and nutrients (Hiraoka, Kimura, Ueda & Tabata, 2003). For example, CollaPlug® is a commercial bio-absorbable sponge made of type I collagen extracted from bovine Achilles tendon (Gruber, Lesli, Ingram, Norton & Hanley, 2004) used for socket augmentation at the time of tooth extraction to reduce alveolar bone loss (Wang & Tsao, 2007). However, despite collagen is considered the ideal framework for porous and biocompatible scaffolds, it has the disadvantage of quick degradation (Chang, et al., 2012) and weak mechanical properties (Yuniki, Ikoma & Tanaka, 2010), as a result CollaPlug® may not be suitable to protect against overextension when used as an internal matrix, together with dental sealers, to repair furcation perforations since is not pressure resistant (Zou et al., 2008). Therefore, for other applications such as bone graft and tissue engineering, collagen scaffolds have commonly been reinforced with hydroxyapatite (HA) (Kane & Roeder, 2012; Kane et al., 2015; Yunoki et al., 2006), a biocompatible ceramic that increases not only the mechanical properties but also the osteoconductivity of the collagen scaffolds

(Rodríguez et al., 2003; Wei & Ma, 2004). However, it should be considered that even though the compression response of materials for applications in dentistry needs to be improved, it has to be in a way that scaffolds continue being compressible and capable of promoting hemostasis. Starch, a carbohydrate formed by a combination of linear (amylose, 20-30%) and branched polymers (amylopectin, 70-80%) (Nair & Laurencin, 2005), is intrinsically biocompatible, biodegradable, water soluble and readily available. It has been used for fabrication of starch-based scaffolds for tissue engineering (Salgado, et al., 2002; Salgado, Coutinho & Reis, 2004; Salgado, Figueiredo, Coutinho & Reiss, 2005) and drug delivery (Shibata, Nishumura, Naruhashi, Nakao & Miura, 2010). Sponges made of starch maintain their shape after absorbing liquid (Bice, MacMasters & Hilbert, 1944), and have been used as absorbable hemostatic agents (Korchin, 1956). Based on this, we hypothesize that if sponges are prepared with collagen, HA and starch; then collagen would provide a framework for the sponges, HA would add stiffness, while the incorporation of starch will keep them compressible and blood-absorbing. Therefore, the combination of HA-starch will lead to hemostatic sponges more structurally robust, which are important characteristics of hemostatic and space filler agents for dental surgery.

The present study sought to improve the mechanical and blood-absorbing properties of collagen sponges, while keeping them compressible, by incorporating blended HA and starch into the scaffolds. The elastic modulus, cell proliferation, and the blood-clotting properties of the sponges were investigated and compared with commercial product, CollaPlug®.

2. Materials and methods

2.1 Raw materials and reagents

The starch used to fabricate the sponges was food grade (94% cornstarch) from Unilever, México, and was used in as received conditions. Bovine femur bone was purchased from a local butcher (San Diego, California, US). Bone was demineralized using EDTA tetrasodium salt, obtained from Spectrum. The isolated collagen was dissolved into acetic acid, glacial, from Fisher Scientific (ACS grade) and compared with type I collagen from calf skin, purchased from Sigma-Aldrich, while the fabricated sponges were compared with CollaPlug® (Zimmer Dental, Indiana, US). Sintered hydroxyapatite (HA) (2-3 μm particle size) was obtained from TransTech. Mouse fibroblast L929 cells were gotten from the American Type Culture Collection (ATCC, CCL-1). RPMI 1640 medium, fetal bovine serum, antibiotic/antimicotic solution, trypsin/EDTA and Hanks' Balanced Salt Solution (HBSS) were purchased from GIBCO. Cell Titer 96 Aqueous One Solution was obtained from PROMEGA.

2.2 Collagen isolation

The collagen used in this study was obtained by demineralization of cortical bovine femur bone using 0.1 M EDTA at 20°C. Bone demineralization was confirmed by measuring the PO_4^{3-} -P released into EDTA solution (Fig. 1a) using an UV/Vis spectrophotometer as described in detail elsewhere (Castro-Ceseña, Novitscaya, Phadke, Varghese & McKittrick, 2013). Briefly, the cleaned cortical bovine bone was cut into pieces (2 cm³) and powdered using a mortar and pestle before immersed into EDTA solution. Demineralization was done for 9 days; while EDTA was refreshed every day. Quality of the isolated collagen (partially soluble) was verified by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) (Fig. 1b) (Castro-Ceseña et al., 2013). In this work, soluble collagen was obtained after solubilization of the demineralized powdered bone into 0.1 M acetic acid, and then concentrating by freeze-drying (Labconco Freeze zone, 1L), referred to herein as acetic acid-solubilized collagen.

Fig. 1. Isolation of partially soluble collagen from bovine femur bone. (a) Confirmation of bone demineralization by measurement of PO_4^{3-} -P released into demineralization solution. (b) SDS-PAGE of the partially soluble isolated collagen from bovine femur bone compared with commercial type I collagen and untreated bone. SDS-PAGE shows that the isolated collagen was not hydrolyzed. Modified from Castro-Ceseña, Novitscaya, Phadke, Varghese & McKittrick, 2013.

2.3 Fabrication of the sponges

All of the sponges were prepared using a concentration of 20 mg/mL of the acetic acid-solubilized collagen (Section 2.2) and 80 mg/mL of HA. The concentration of starch varied from 40 to 420 mg/mL, but after preliminary evaluations (results not shown), 100 and 200 mg/ml of starch were chosen for this study. For simplicity, the sponges prepared will be identified as: 1:0:0, 1:4:0, 1:4:5 and 1:4:10 (collagen:HA:starch, by weight) from this point forward, which means the times that collagen concentration (20 mg/mL) was doubled with HA and/or starch.

Therefore, while collagen and HA concentrations were fixed for all the sponges, the mass of starch was increased but, sponges with form of cylinder of 1 cm high x 0.5 cm diameter were obtained for all of the collagen:HA:starch samples, thus compression tests would be performed under the same conditions, and elastic modulus data would be attributed to the addition of HA-starch blends. Sponges were obtained by adding a solution of well mixed collagen, HA and starch into a 96 well plate (Corning, 3526), and then freeze-drying (Labconco Freeze zone, 1L) for 24 h. Each sponge was taken out from the 96 well plate and then placed into aluminum foil trays. Sponges were cross-linked by heat-dehydration (NAPCO vacuum oven) at 145 °C and 100 kPa for 48 h, which consists on removal of water from proteins to form interchain cross-links as a result of condensation reactions either by esterification or amide formation. Amino acid residues such as aspartic acid, glutamic acid, serine, threonine, arginine and lysine, which constitute ~745 of the 3156 residues on collagen, may be involved in those condensation reactions (Yannas & Tobolsky, 1967; Weadock, Olson & Silver, 1983-84).

2.4 Sponge microstructure

The surface structure of the sponges was imaged using scanning electron microscopy (SEM), before and after cell proliferation assays. The seeded sponges were washed three times with 1X PBS to completely remove the culture medium. Sponges were then stored at 4 °C with 1 mL of 4% formaldehyde diluted in 1X PBS, until use. Before SEM imaging, sponges were dehydrated with a graded series of ethanol, and then subjected to critical point drying to avoid shrinkage. Samples transverse sections were coated with iridium using an Emitech K575X ion sputter coater (Quorum Technologies Ltd, West Sussex, UK) and then imaged by SEM (FEI-XL30, FEI Company, Oregon, US) at a voltage of 3 kV.

2.5 Compression testing

Compression tests were performed to all of the fabricated sponges and CollaPlug® (each in triplicate). Samples were rehydrated in 1X HBSS for 0.5 h at room temperature prior to testing

and were kept soaked into 1X HBSS during loading. Sponges were loaded at a strain rate of $1 \times 10^{-3} \text{ s}^{-1}$ to 75% strain using a universal testing machine with a 500 N load cell (3342 Single Column System, Instron, Massachusetts, US). The small displacements were measured using an external deflectionometer SATEC model I3540 (Epsilon Technology Corp., Wyoming, US). Elastic modulus was calculated from the slope of the stress-strain curve in the linear region over a 2% strain range within all data between 0 and 10% strain.

2.6 Cell attachment assay

2.6.1 Cell culture

Mouse fibroblast L929 cells were cultured in a cell culture dish with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1X of antibiotic/antimycotic solution in humidified atmosphere, 5% CO₂ air, at 37 °C, until confluence was 80%. The cells were detached from the plate using 1 mL of trypsin/EDTA, and then incubated for 3 min at 37 °C. The cells solution obtained was centrifuged at 3000 *g* for 5 min. After this, cells were counted using trypan blue and a TC10 Automater Cell Counter (BIORAD, 145-0001), resulting in 99% of live cells.

2.6.2 Cell seeding

CollaPlug® collagen sponges were used as a positive control for all of the experiments. Sponges were sterilized by 254 nm UV (AB) lamp for 10 min in a laminar flux chamber (Thermo Scientific Forma Series II, 3110) and placed, in triplicate, in a 24 well culture plate (Corning, 3526). Sponges were soaked three times into 0.1 mL of RPMI supplemented medium (10% FBS and 1X antibiotic-antimycotic), with 1×10^5 cells per well. The culture plate was incubated in humidified, 5% CO₂ air atmosphere at 37°C. Next, the sponges were washed three times with sterile 1X PBS, and then cut into two pieces (one for cell proliferation assays, and one for SEM imaging). The scaffolds were evaluated after 2 and 5 days of contact with cells. The RPMI supplemented medium was refreshed every 48 h.

2.6.3 Cell viability

After each incubation time (2 and 5 days), the sponges were washed three times with sterile 1X PBS to completely remove the culture medium. Afterwards, the sponges were transferred to a new 24 well culture plate. Sponges were then treated with 1 mL of 0.25% trypsin/EDTA for 3 min in a humidified, 5% CO₂ air atmosphere at 37 °C. After incubation, the sponges were washed with trypsin/EDTA solution and transferred to a 15 mL tube with 3 mL of fresh RPMI

supplemented culture medium, prior centrifugation for 5 min at 3000 *g*. Each pellet was resuspended in fresh 0.1 mL of RPMI supplemented medium and transferred to a new 96 well plate. Finally, 0.020 mL of Cell Titer 96 Aqueous One Solution (MTS assay) was added per well before incubation in a humidified, 5% CO₂ air atmosphere for 2 h. The absorbance was read in an ELISA plate reader (BioTek, Epoch) at 495 nm. Results were presented as the percentage of viable cells normalized with respect to the commercial control CollaPlug®, since one of the goals of this work was to investigate and compare the mechanical and in vitro properties of collagen:HA:starch sponges with respect to a commercially available collagen sponge used in dentistry, as the sponges prepared in this study are intended to be used for dental applications. The amount of absorbance is directly proportional to the number of living cells in culture attached to the sponges; therefore sponges that showed higher absorbance were chosen for blood-clotting assay.

2.7 Blood-clotting assay

Blood-clotting and preservation of erythrocytes integrity capability of the collagen:HA:starch sponges were analyzed and compared with commercially available CollaPlug®. Blood samples were obtained from healthy volunteers by venipuncture under aseptic technique, and collected in acid citrate dextrose (ACD) anticoagulant vacutainer tubes. Sponges were cut to obtain pieces of 0.5 cm high x 0.5 cm diameter and placed in a 12 well plate. Clotting reaction started when 0.85 mL of 0.2 M CaCl₂ was added to the total of blood collected. The activated blood (0.1 mL) was carefully infiltrated twice through the sponges. The samples were then incubated, in triplicate, at room temperature for 5 and 15 min, and then were incubated for 5 min with 3 mL of distilled water. Samples were centrifuged at 300 *g* during 5 min and 0.2 mL of supernatant from each sample, were transferred to a 96 well plate. The red blood cells that cannot be trapped into thrombi would be lysed. Hemoglobin released into water was read at 540 nm (BioTek, Epoch) using water as control as previously described (Huang et al., 2003; Sudheesh Kumar et al., 2010; Sun et al., 2009).

2.8 Analysis of data

Statistical analysis of results (elastic modulus, cell attachment and blood-clotting) was done by an analysis of variance (ANOVA) of the main effects. Statistical significance between each of the immobilization conditions was determined by Tukey's *a posteriori* test, and significance was determined at $p < 0.05$. Origin Pro 8.5, Statistics 8 and Microsoft Excel were used for processing of data and statistical analysis of results.

3. Results and discussion

3.1 Structural characteristics

Every gram of untreated ground bovine cortical bone yielded 0.125 ± 0.017 g of partially soluble collagen flakes after nine days of EDTA demineralization (Fig. 2a) but completely soluble collagen (0.028 ± 0.001 g) was obtained only after concentrating, by freeze-drying, the acetic acid-solubilized fraction of the collagen flakes (Fig. 2b). Therefore, collagen was extracted with a yield of 2.8% on the basis of the dry weight, a considerably good yield when taking into account, that 1.6% (wet weight) of yield was obtained when collagen was extracted from bone of bigeye snapper (*Priacanthus tayenus*) (Kittiphattanabawon, Benjakul, Visessanguan, Nagai & Tanaka, 2005).

Fig. 2. Photographs showing (a) flakes of partially soluble collagen obtained by EDTA demineralization of bovine femur bone and, (b) completely soluble collagen obtained after freeze-drying the acetic acid-solubilized fraction of the collagen flakes obtained in (a). (c) This collagen was used to fabricate the collagen:HA:starch biocomposites.

The acetic acid-solubilized collagen obtained was used to prepare the collagen:HA:starch biocomposites (Fig. 2c). The morphologies of collagen sponges with different concentrations of HA and starch was analyzed by SEM (Fig. 3). SEM micrographs of the transversal section of the sponges showed that morphology changed with concentration of incorporated starch, as previously shown for sponges prepared with starch (Bice et al., 1944). Although SEM does not provide quantitative information about porosity, it can be seen that sponges 1:0:0 and 1:4:0

(collagen:HA:starch by weight), exhibited semispherical, irregular and interconnected pores between 150 and 200 μm , similar to those found in CollaPlug® (Figs. 3a-c); while pore size reduction ~ 75 and ~ 25 μm occurred in sponges 1:4:5 and 1:4:10, respectively, as starch concentration increased from 100 to 200 mg/mL (Figs. 3d-e). **These sponges had a rougher-granular microstructure corresponding to starch, since is apparent that concentration of the granules increased with starch concentration. In addition, the granules in the sponges had a size of ~ 15 μm like native (unmodified) starch granules (Cho & Lee, 2002). One of the goals of this work was to improve the elastic modulus of collagen sponges, which can be achieved by chemically modifying the starch used, since its mechanical properties are weak. However, the prepared sponges should keep being compressible. To accomplish this, HA blended with native, chemically unmodified, starch granules was used, producing the granular microstructure of the starch containing sponges (Figs. 3d-e).**

Fig. 3. Representative SEM micrographs showing the transversal section of the collagen:HA:starch sponge. (a) CollaPlug®, used as commercial control, (b) 1:0:0, (c) 1:4:0, (d) 1:4:5, (e) 1:4:10 collagen:HA:starch by weight. The structural characteristics of the sponges changed as starch was incorporated into the biocomposites. Semispherical and interconnected pores are visible in sponges 1:0:0 and 1:4:0 collagen:HA:starch by weight, similar to those found in CollaPlug®. Pore size reduction was observed as concentration of starch increased in sponges.

3.2 Compression behavior of the sponges

Similar to all of the biocomposites, collagen sponges with HA and starch exhibited compressive stress-strain curves characteristic of elastic foams (Fig. 4a) however, differences such as collagen source, addition of HA or concentration of starch, led to structural characteristics that directly affected the mechanical response of the sponges. CollaPlug®, the commercial control, had an elastic modulus of 1.45 ± 0.20 kPa, while sponge 1:0:0 had a value of 18.30 ± 2.00 kPa, i.e. 12.5 times higher ($P < 0.05$) than that of the commercial control. CollaPlug® is made of type I collagen extracted from bovine Achilles tendon (Gruber et al., 2004), while sponges 1:0:0 (collagen:HA:starch, by weight) were prepared with collagen isolated from bovine bone (section 2.2). The collagen isolated in this work did not contain mineral after treatment with EDTA (Fig. 1a), then the increased elastic modulus can be attributed to factors other than remaining mineral. Previous studies on type I collagens isolated from different sources of the same organism, for example skin and bone from bigeye snapper, have showed that although both are type I collagens, there are slight differences on their enzymatically digested peptide maps, indicating differences in amino acid composition, therefore in their structural conformation (Kittiphattanabawon, Benjakul, Visessanguan Nagai & Tanaka, 2005). Moreover, it has been proposed that modification and rearrangement of a few amino acid motifs on spider silk proteins, directly correspond to the mechanical properties exhibited by those proteins (Hayashi, Shipley & Lewis, 1999). Based on this, it is plausible to attribute the difference in the elastic modulus between CollaPlug® and sponges 1:0:0 (collagen:HA:starch) to the different sources of collagen, i.e. tendon or bone, both from bovine. The incorporation of HA also influenced the compression behavior, as expected since HA increases the stiffness and hardness (Kane & Roeder, 2012). The addition of HA increased the elastic modulus from 18.30 ± 2 kPa to 23.04 ± 0.5 kPa for 1:0:0 and 1:4:0 (collagen:HA:starch), respectively ($P < 0.05$) (Fig. 4b). However, the best elastic moduli were obtained when starch was added to the biocomposites, 31.32 ± 2 and 48.76 ± 8 kPa for sponges 1:4:5 and 1:4:10 (collagen:HA:starch, by weight) respectively. Thus elastic modulus of 1:4:10 (collagen:HA:starch) sponges was found to be two times higher with respect to 1:4:0 ones ($P < 0.05$). HA adheres strongly to collagen and starch because of the polar nature of these polymers. In this case, collagen provided a framework to the sponges, while starch, the polymer present in mayor amount, could act like a dispersing agent, leading to a more homogeneous distribution of the HA through the sponges, which improves its mechanical properties. However, the decreased porosity (increased density) and the reduction of pore sizes with increasing of starch concentration (Figs. 3 d,e), were the main reasons for the elastic modulus increment. Thus, porosity, pore size and internal structure of sponges are the

main factors that directly affect the mechanical performance of these materials. The relative elastic modulus (E^*/E_s) and relative compressive strength (σ^*/σ_s) are directly related to the fraction of porosity (ϕ) in a porous material (Gibson & Ashby, 1997),

$$\frac{E^*}{E_s} \approx (1 - \phi)^n \quad (1)$$

where E^* is the measured elastic modulus of the porous material, E_s is the elastic modulus of the completely dense solid and n usually ranges between 1-3. This indicates that the elastic modulus is strongly correlated with the amount of porosity in the porous material.

Fig. 4. Compression behavior of the sponges synthesized. (a) Representative stress-strain curve for the compression of collagen:HA:starch sponges at 1:4:5 collagen:HA:starch by weight. The elastic modulus was calculated as the slope over a 2% strain range within all data between 0 and 10% strain. (b) Mean elastic modulus of the collagen sponges reinforced with HA and starch at 1:0:0, 1:4:0, 1:4:5 and 1:4:10 collagen:HA:starch by weight, while CollaPlug® was used as a control ($n = 5/\text{group}$). Error bars show one standard deviation. Sponges prepared with the isolated collagen from bovine bone alone (sponges 1:0:0) significantly increased elastic modulus compared with CollaPlug® ($*P < 0.05$, sponge 1:0:0 > CollaPlug®, Tukey's HSD). Starch improved the elastic modulus of the sponges compared with using only HA ($\square P < 0.05$, sponges 1:4:5 and 1:4:10 > sponges 1:4:0, Tukey's HSD). All of the compression tests were performed under wet condition (1X HBSS).

3.3 Cell viability

After the results obtained from the compression tests, analysis of cell viability was done on the sponges that contained the blended starch, HA and collagen, and the results were

compared with CollaPlug® (as commercial control) which is pure collagen, and our collagen sponge (scaffold 1:0:0, collagen:HA:starch, by weight). Thus, mouse fibroblasts L929 cells were put into contact with the scaffolds and incubated for 2 and 5 days. The MTS assay was used to **measure cell viability**, which is a colorimetric assay that indirectly measures the metabolic activity of **living** cells based on the reduction of MTS reagent to brown formazan product when incubated with viable cells (Tuzlakoglu et al., 2005). Therefore, the amount of the absorbance read was directly proportional to fibroblasts cells that **kept viable** on the sponges. **A culture-grade polystyrene well plate is generally used as positive control in the MTS assay. However, the results obtained here were normalized with respect to CollaPlug®. A sponge (3D structure) resembles biological models more than 2D structures, i.e. the polystyrene well plate. This allowed comparison of our sponges with structures that are more similar to those found in nature. Moreover, the culture-grade polystyrene well plate promotes a higher cell growth rate compared with 3D structures, because of factors such as distribution of nutrients, which is different in the polystyrene plate monolayer and the 3D sponge. Although collagen may have some degree of cytotoxicity because of some physicochemical phenomena of degrading collagen-based material are suspected to be a cause of oxidative stress (Yamada et al., 2010), our results demonstrate that other factors such as the microstructure of the scaffolds directly influence the viability of the cells seeded on the sponges. In this case, the higher absorbance read, interpreted as higher number of cells attached to the scaffolds, was measured in CollaPlug® compared with sponge 1:0:0 ($P < 0.05$) at day 5 (Fig. 5a). Both sponges are made of type I collagen, and then this difference on cell attachment can be attributed to the structural characteristics of the scaffolds. Analysis of the microtopography of the sponges showed that surface of CollaPlug® was rougher than that of sponge 1:0:0 (collagen:HA:starch, by weight) (Figs. 3a-b). A rough surface is been previously suggested to provide sites for protein adhesion and cell attachment (Kane & Roeder, 2012), which could be the reason for the higher **cell viability** found in CollaPlug® compared with our collagen sponge. However, the highest content of cells attached to sponges was found for those that contained starch in addition to collagen and HA at day 5 of incubation, i.e. sponges 1:4:5 and 1:4:10 compared with CollaPlug® and sponges 1:0:0 (collagen:HA:starch) ($P < 0.05$) (Fig. 5a). The microtopography of these scaffolds showed that sponges 1:4:5 and 1:4:10 were rougher because of the granules of the incorporated starch (Figs. 3d-e), which directly influenced the **cell viability** by increasing the content of fibroblasts on the scaffolds. Moreover, fibroblasts on collagen:HA:starch sponges had a flattened morphology (Fig. 5b), indicating good contact between fibroblasts and sponges, directly related to the biocompatibility of the scaffolds (Nath, Tripathi & Basu, 2009). **Although****

there is no evidence so far supporting that starch per se stimulates cell viability, our results indicate that increased cell viability is attributed to starch, since collagen and HA concentrations were kept constant in all the sponges, while cell viability increased on those containing starch, which can be attributed to its granular microstructure. However, more analyses need to be done to clarify the factors that contributed to the increased cell viability.

It was found that the incorporation of starch to the sponges increased the cell viability. Although, as dental fillers, these materials should prevent volume reduction after tooth extraction, an ideal graft material placed in extraction sockets should also promote healing, i.e. bone formation, to allow initiation of osteogenesis (Serino, Rao, Iezzi & Pattelli, 2008).

Fig. 5. Viability of fibroblast L929 cells on sponges. (a) Cell viability (MTS assay) of mouse fibroblast L929 cells cultured in collagen:HA:starch sponges at days 2 and 5 ($n = 3/\text{group}$). Error bars show one standard deviation of the mean. Incorporation of starch into the sponges increased cell viability at days 2 and 5 compared with those seeded on CollaPlug® ($*P < 0.05$, sponges 1:4:5 and 1:4:10 > CollaPlug®, Tukey's HSD) and cells seeded on pure collagen sponge ($\square P < 0.05$, sponges 1:4:5 and 1:4:10 > 1:0:0, Tukey's HSD). (b) Representative SEM micrograph showing fibroblast L929 cells attached to sponge 1:4:10 collagen:HA:starch by weight.

3.4 Blood-clotting

From cell proliferation results, it was decided to test sponges 1:4:5 and 1:4:10 (collagen:HA:starch, by weight) because of the high cell proliferation obtained, and compare to the results for the commercial CollaPlug®. The blood-clotting assay performed measures the

hemolyzed red blood cells that were not trapped in the clot formed on the sponges (Sudheesh Kumar et al., 2010), therefore the higher the absorbance read, the lower the clotting efficiency of the scaffold in terms of clotting rate. The results showed that during the first 5 min of contact with blood, CollaPlug® clotted blood more rapidly than sponges with composition 1:4:5 and 1:4:10 ($P < 0.05$) (Fig. 6). We expected that combination of two hemostatic agents with high efficacy, collagen and starch, would result in an improved clotting rate when compared with pure collagen sponges. However, two factors should be considered: 1) that sponge with composition 1:4:5 contained starch with concentration 5 times the concentration of collagen, therefore it is reasonable that starch would direct the blood-clotting response of the scaffolds and, 2) that collagen and starch promote blood-clotting in different ways. While collagen is a protein that participates in platelet activation after injury by supporting the adhesion, spread and activation of platelets to form an aggregate (Farndale, Sixma, Barnes & Groot, 2004); the granules of starch act as molecular sieves that locally dehydrate blood by absorbing its fluids and molecular components, concentrating platelets and other serum proteins on the surface of the scaffolds, which activates the clotting process (Ereth et al., 2008). Therefore, CollaPlug® (pure collagen) initiated blood-clotting more rapidly than starch, i.e. sponges 1:4:5 (collagen:HA:starch, by weight). But the blood-clotting response of the sponges was improved when starch was doubled from 5 to 10, i.e. sponge 1:4:10 clotted more blood than sponge with composition 1:4:5 ($P < 0.05$) after 5 min of contact with blood ($P < 0.05$) (Fig. 6). These results indicate that blood-clotting depended on starch concentration. After 15 min of incubation with blood, sponges 1:4:10 (collagen:HA:starch, by weight) released less hemoglobin to the medium (lower absorbance) compared with CollaPlug® and sponges with composition 1:4:5 (Fig. 6). Moreover, the hemoglobin released by CollaPlug® was doubled from 5 to 15 min of incubation with blood, while the hemoglobin released by sponges with composition 1:4:10 was from 0.35 at 5 min to 0.39 units of absorbance at 15 min of incubation, i.e. a minimum change from 5 to 15 min after exposure with blood compared with CollaPlug® (Fig. 6). We speculate that over time CollaPlug® got saturated with blood, not allowing the formation of more clots, and then the membrane of more erythrocytes was disrupted and hemoglobin was released because there was no more substrate to form a clot, which was measured as an increased absorbance from 5 to 15 min of incubation. However, sponges 1:4:10 (collagen:HA:starch, by weight) absorbed blood more slowly than CollaPlug® during the first 5 min of incubation, and after 15 min the sponge 1:4:10 was not totally saturated, allowing the formation of more clots, resulting in less hemoglobin released compared with CollaPlug®.

Fig. 6. Blood-clotting results for the collagen:HA:starch sponges after 5 and 15 min of incubation in blood ($n = 3/\text{group}$). Error bars show one standard deviation of the mean. CollaPlug® clotted blood more quickly than the synthesized sponges during the first 5 min of incubation in blood ($*P < 0.05$, CollaPlug® > sponges 1:4:5 and 1:4:10, Tukey's HSD). However, after 15 min of contact with blood, sponges 1:4:10 (collagen:HA:starch by weight) showed higher blood-clotting capability, i.e. lower absorbance, compared with CollaPlug® ($*P < 0.05$, sponge 1:4:10 > CollaPlug®, Tukey's HSD). Sponges with composition 1:4:10 showed better blood-clotting characteristics than 1:4:5 at any incubation time evaluated ($\square P < 0.05$, sponge 1:4:10 > 1:4:5, Tukey's HSD).

4. Conclusions

Collagen:HA:starch-based sponges were synthesized and evaluated in terms of their compression, cell proliferation and blood-clotting properties. The highest elastic moduli were obtained when blended HA-starch was added to the sponges. **The decreased porosity (increased density) and the reduction of pore sizes with increasing of starch concentration were the main reasons for the elastic modulus increment, thus making sponges more structurally robust.**

The modified microstructure and surface area influenced the cell proliferation, as a consequence of the incorporated starch, by increasing the number of cells attached to the sponges.

Sponges 1:4:10 (collagen:HA:starch) maintained their blood-clotting capability with almost no change from 5 to 15 min after contact with blood, while the commercial collagen control,

CollaPlug®, diminished to about half its capacity to absorb blood and form clots when evaluated under the same conditions after 15 min of incubation with blood.

Based on the results obtained, incorporation of starch into the sponges in a ratio of 1:4:10 (collagen:HA:starch, by weight), not only increased the elastic modulus of the collagen-HA sponges, but also increased the cell proliferation, as well as the blood-clotting capability of the scaffolds. These characteristics make collagen:HA:starch sponges a good choice for further evaluations (such as degradation studies) to be used in dentistry.

Acknowledgements

We thank Ryan Anderson of CalIT2, UCSD, for helping with SEM imaging. The University of California Institute for Mexico and the United States (UC MEXUS-CONACyT) is acknowledged for the postdoctoral fellowship provided to Ana B. Castro-Ceseña. This work has been funded by the National Science Foundation, Division of Materials Research, Ceramics Program (Grant# DMR1006931) and the Biomaterials Program (Grant# DMR 1507978).

The authors declare no conflict of interest.

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