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Bio-inspired *Aloe vera* sponges for biomedical applications

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ABSTRACT

Chemical composition and biological properties of *Aloe vera* (AV), a tropical plant, explain its potential use for cosmetic, nutritional and biomedical applications. AV gel present in AV leaves is rich in several compounds, nutrients and polysaccharides. This work proposes using AV gel complex structure and chemical composition, associated with freeze-drying, to produce sponges. To increase the structures stability in aqueous media, a thin coating of gellan gum (GG), was applied onto AV gel. AV-based sponges showed a heterogeneous porous formation, interconnected pores and good porosity (72–77%). The coating with a GG layer onto AV influenced the stability, swelling behavior and mechanical properties of the resulting sponges. Moreover, sponges provided the sustained release of BSA-FTIC, used as a model protein, over 3 weeks. Also, *in vitro* cell culture studies evidenced that sponges are not cytotoxic for a mouse fibroblast-like cell line. Therefore, developed AV-based sponges have potential use in biomedical applications.

1. Introduction

Worldwide, there is a need for the search for new therapies and drugs due to the incidence of diseases such as cancer and diabetes. As such, the use of plants as a natural resource of bioactive phytochemical compounds may be useful for developing new formulations and strategies to enhance life quality. It is recognized that some plant extracts contain compounds and polysaccharides, which display anticancer, antioxidant, antibacterial and antiviral activities. *Aloe vera* (AV), a tropical plant belonging to the *liliaceae* family, is known to be the oldest medicinal plant in Nature (Gage, 1996). AV plant *per se* is an unique resource of the natural compounds, nutrients and polysaccharides with several bioactivities (Boudreau & Beland, 2006; Hamman, 2008; Reynolds & Dweck, 1999). This chemical diversity has encouraged its utilization in cosmetic formulations, food supplements and medical devices (Cole & Heard, 2007; Hamman, 2008; Rodriguez Rodriguez, Darias Martin, & Diaz Romero, 2010; Silva, Caridade, Mano, & Reis, 2013; Silva, Popa, et al., 2013). By its turn, the properties of AV gel, contained in the inner parts of the fresh AV leaves (Hamman, 2008) have anti-inflammatory, antioxidant, antiviral and antibacterial action. These properties are associated to its complex composition. AV gel has also been useful to design and create functional films, microspheres and sponges. Most of these biomaterials have been prepared by combining AV gel with bacterial cellulose, chitosan, alginate, and gelatin (Chen, Wang, & Weng, 2010; Pereira, Tojeira, Vaz, Mendes, & Bartolo, 2011; Saibuatong & Phisalaphong, 2010; Silva, Caridade, et al., 2013; Silva, Popa, et al., 2013). For instance, Silva, Caridade, et al. (2013) and Silva, Popa, et al. (2013) reported that a small portion of AV gel into chitosan provided blended membranes with adequate roughness, degradation rate, wettability and mechanical properties to be used as wound dressing materials. Due to the wide use of AV, a high number of possibilities are still open to investigation. Therefore, in the present work, the exploitation of AV gel, in the development of sponges using freeze-drying technique was evaluated. It is well known that freeze drying is one of the most popular technique for producing three dimensional (3D) porous structures based on natural or synthetic polymers (Reys et al., 2011; Silva et al., 2008). This technique is based on the formation of ice crystals that induce porosity through ice sublimation and desorption (Mano et al., 2007); that could be useful for increasing the shelf-life of Aloe components (Krokida, Pappa and Agalioti, 2011). To increase the stability of the structures in aqueous media, a thin coating layer of gellan gum (GG), was applied onto AV gel. GG is a well-known water soluble, biocompatible polysaccharide that could be used as an adhesive to apply seasoning to the surfaces of foods (Oliveira,

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Santos, et al., 2010). In addition, GG has been proposed for tissue engineering applications (Oliveira, Martins, et al., 2010; Oliveira, Santos, et al., 2010; Silva-Correia et al., 2013). We hypothesized that the developed AV-based sponges could represent a natural therapeutic strategy, where the natural biological activity of AV may act in different stages of the tissue regeneration. To evaluate the potential use of AV-based sponges as a carrier for therapeutic molecules, the sponges were labeled with a model protein, namely bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FTIC). Furthermore, the studies presented in this article focus on understanding the morphological features, mechanical properties and cellular response of the AV-based sponges.

2. Materials and methods

2.1. Materials

Fresh whole *A. vera* (*Aloe barbadensis Miller*) leaves, obtained from a Portuguese botanic shop, were used as the raw material in all experiments. The studied leaves, between 30 and 40 cm of length, corresponded to 4-year old plants. Low-acyl gellan gum (GG, Gelzan^{TM}) was purchased from Sigma–Aldrich Co., USA. All other reagents were obtained from Sigma–Aldrich Co. (USA), unless otherwise indicated.

2.2. Methods

2.2.1. Preparation of the Aloe vera-based sponges

All whole *A. vera* leaves were washed with distilled water to remove dirt from the surface. The skin was separated from the parenchyma using a scalpel-shaped knife. The AV filets were extensively washed with distilled water to remove the exudates from their surfaces. AV filets were cut in small cubes (diameter 0.8 cm). Gellan gum (GG) powder was mixed with distilled water under constant stirring at room temperature to obtain a concentration of 1% (w/v). The solution was heated to 90 °C during 20–30 min. Afterwards, the temperature was progressively decreased to 50 °C (Oliveira, Martins, et al., 2010). Further, the molded AV gels were immersed into GG solution during 30 s, in order to create a thin layer on the material. After that, AVG matrices were rinsed with phosphate buffer solution (PBS). Then, both matrices (AV and AVG) were stabilized at 65 °C for 15–20 min, and at 4 °C for 5 min, followed by freezing at −80 °C overnight and freeze-drying for 3 days. AV sponges were also prepared without any GG coating. The identification of the AV-based sponges was AVG and AV, for those prepared with and without GG coating, respectively.

2.2.2. Bovine serum albumin (BSA) incorporation

The AV-based sponges were incubated into bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FTIC) at a concentration of 2 mg/ml overnight at 4 °C to avoid the gel oxidation. Afterwards, the samples were frozen at −80 °C and submitted to freeze-drying for 3 days.

3. Characterization

3.1. Scanning electron microscopy (SEM)

Samples of the freeze-dried hydrogels were observed by a Leica Cambridge S360 Scanning Electron Microscope. The materials were fixed by mutual conductive adhesive tape on aluminum stubs and covered with gold palladium using a sputter coater.

3.2. Micro-computed tomography (μ-CT)

The microstructure of the sponges was evaluated using a high-resolution μ-CT Skyscan 1072 scanner (Skyscan,Kontich, Belgium) with a resolution pixel size of 6.59 μm and integration time of 1.9 s. The X-ray source was set at 32 keV and 143 μA. Approximately 500 projections were acquired over a rotation range of 180° with a rotation step of 0.45°. Data sets were reconstructed using standardized cone-beam reconstruction software (NRecon v1.4.3, SkyScan). The output format for each sample was 500 serial 1024 × 1024 bitmap images. A representative data set of 500 slices was segmented into binary images with a dynamic threshold of 57–255 (gray values). The same representative volume of interest (VOI) was analyzed for all the samples. These data sets were used for morphometric analysis (CT Analyser, v1.5.1.5, SkyScan) and to build the 3D models (ANT 3D creator, v2.4, SkyScan).

3.3. Swelling behavior

Swelling tests were performed by immersing the sponges in different buffer solutions (pH 5, 7.4 and 10) up to 360 min. The swollen sample weights were measured after removing excess surface water by gently tapping the surface with filter paper. The percentage of water uptake was calculated using Eq. (1), where w_s is the weight of the swollen sample and w_d is the weight of the dry sample. Each experiment was repeated three times, and the average value was considered to be the water uptake value.

$$\text{Water uptake}\,(\%) = \left[\frac{W_s - W_d}{W_d}\right] \times 100 \quad (1)$$

3.4. Stability

The AVG matrices (6.5 mm diameter, 4.5 mm height) were immersed in phosphate buffer solution (PBS) at 37 °C for up to 30 days. Samples were collected at different time points, washed gently with distilled water, dried at 40 °C and weighed. Percentage loss in weight at respective time points was calculated.

3.5. In vitro BSA release

BSA-FTIC-loaded sponges were weighted and suspended in 3 ml of PBS. The samples were immersed in a thermostatic water bath at 37 °C and 60 rpm. Aliquots of 300 μl were withdrawn at predetermined time intervals, and the same volume of fresh buffer was added to the suspension. The samples were analyzed by UV-spectroscopy at 280 nm and 495 nm to eliminate the contribution of the FITC absorption at 280 nm in a microplate reader (Synergy HT, Bio-Tek Instruments, USA). The results presented are the average of three measurements. Calculations on the amount of drug released took into account the replacement of aliquots with fresh medium. The concentration of BSA-FTIC was calculated using a standard curve (concentrations ranging from 0.0 to 1 mg/mL), relating the amount of BSA-FTIC with the intensity of light absorbance.

3.6. Dynamical mechanical analysis (DMA)

The viscoelastic measurements of the AV sponges before and after GG coating were performed using a TRITEC2000B DMA from Triton Technology (UK), equipped with a compression mode. The matrices were always analyzed in hydrated conditions through immersion of the samples in a Teflon reservoir containing PBS, used during all analysis period. After equilibration at 37 °C, the DMA spectra were obtained during a frequency scan between 0.1 and 10 Hz. The experiments were performed under constant strain

amplitude (30 μm). The average value of four tests was reported for each sample.

3.7. Cytotoxicity tests

Before testing, the samples were sterilized under an ethylene oxide atmosphere. To assess the possible cytotoxicity of the samples, extracts (leachables) of both sponges (AV and AVG) were evaluated following MEM extraction tests (72 h), according to ISO-10993-5 guidelines (ISO/10993, 1992). In MEM extraction tests, both materials and the control (latex as positive control) were extracted by incubation in culture medium (the same used for cell culture) for 24 h at 37 °C and 60 rpm (in thermostatic bath) (Gomes, Reis, Cunha, Blitterswiijk, & de Bruijn, 2001). After the defined period of incubation, the resulting solution was removed and filtrated, obtaining the extraction fluid. For these tests a mouse fibroblast-like cell line, L929 cells, (European Collection of Cell Cultures-ECACC, UK) at passage 16 was used. L929 cells were cultured in basic medium: Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) with phenol red and supplemented with 10% fetal bovine serum, FBS (Gibco, UK) and 1% antibiotic/antimycotic (A/B, Gibco, UK) solution and incubated at 37 °C in an atmosphere containing 5% CO_2 until achieving 90% confluence. Then, a cell suspension was prepared with a concentration of 1×10^4 cells ml^{-1} and seeded on extracts in 24-well plates and incubated for 72 h. The metabolic activity of L929 cells in contact with the extracts of the sponges was assessed using an AlamarBlue assay. For this assay, an Alamar Blue solution (AlamarBlue®Cell Viability Assay Protocol, Life Technologies) was prepared, added to the medium and incubated for 4 h at 37 °C. The optical density (OD) was read at 570 and 600 nm in a multiwell microplate reader (Synergy HT, BioTek Instruments). The L929 cell viability (%) was determined for each extract and compared to tissue culture polystyrene (TCPS) wells, used as a negative control of cell death. Latex extracts were considered as a positive control of cellular death.

3.8. Statistical analysis

All quantitative experiments were run in triplicate and results were expressed as mean ± standard deviation for $n = 3$. Statistical analysis of the data was conducted using two-way ANOVA with Bonferroni's posthoc comparisons by means of using the GraphPadPrism version 5.0 for Windows (GraphPad Software, San Diego, http://www.graphpad.com). Differences between the groups with $p < 0.05$ were considered to be statistically significant.

4. Results and discussion

4.1. Morphological features

For construction of AV-based sponges (AV and AVG), the AV gel was molded in cylindrical shapes (0.8 cm diameter, Fig. 1a). Even though the matrices showed good mechanical stability, the stabilization of AV gel should be considered before use. Then, the stabilization of AV-based structures (AV and AVG) was performed by applying high temperature (65 °C) for a short period of time (15–20 min). This process will preserve the biological activity of the AV gel. GG is a linear anionic polysaccharide that forms thermoreversible gels, and it has demonstrated non-cytotoxicity in different studies (Oliveira, Santos, et al., 2010). In our work, a thin layer of GG solution (1 wt%) was applied on molded AV gel. Since the gelation of GG solutions is influenced by the presence of cations (Oliveira, Martins, et al., 2010), the matrices were rinsed with PBS which allowed the formation of a three dimensional network on the AV gels, increasing their stability and mechanical properties.

Both developed AV an AVG sponges showed a heterogeneous porous formation distributed in the matrix (Fig. 1b–d). Moreover, the freeze-drying process allowed retaining properly the biological activity of the gel and preserving the cell wall polysaccharides network of AV. It is also increased the shelf-life of the produced sponges (Krokida et al., 2011).

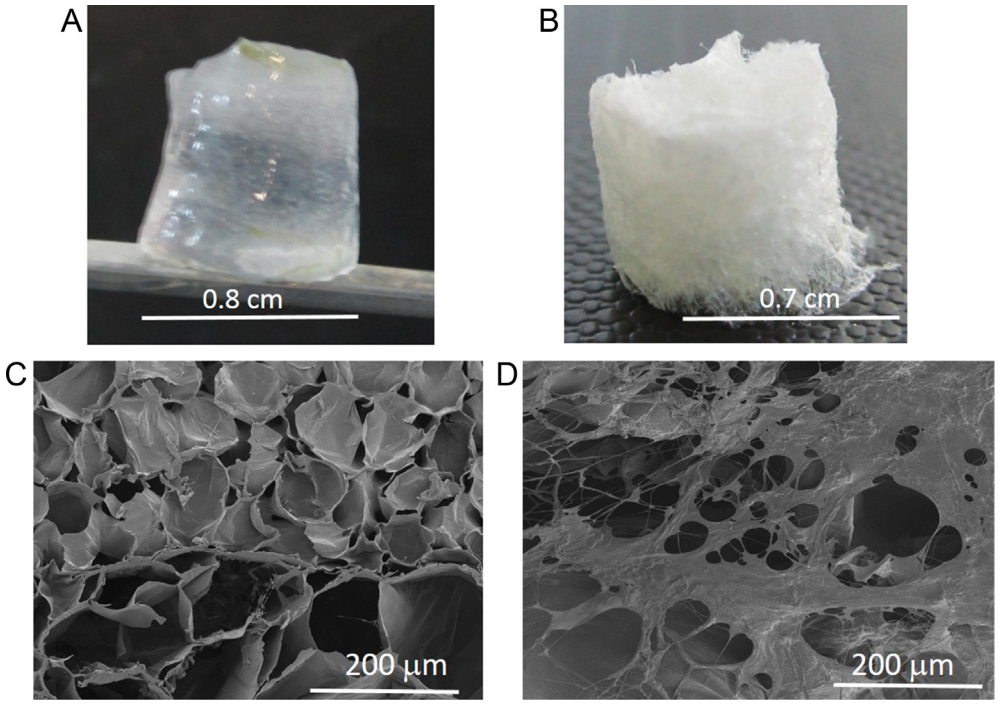

Fig. 1. Photographs of the AV gel (A), AVG sponge (B) and representative SEM image of the surface of the AV (C) and AVG sponges (D).

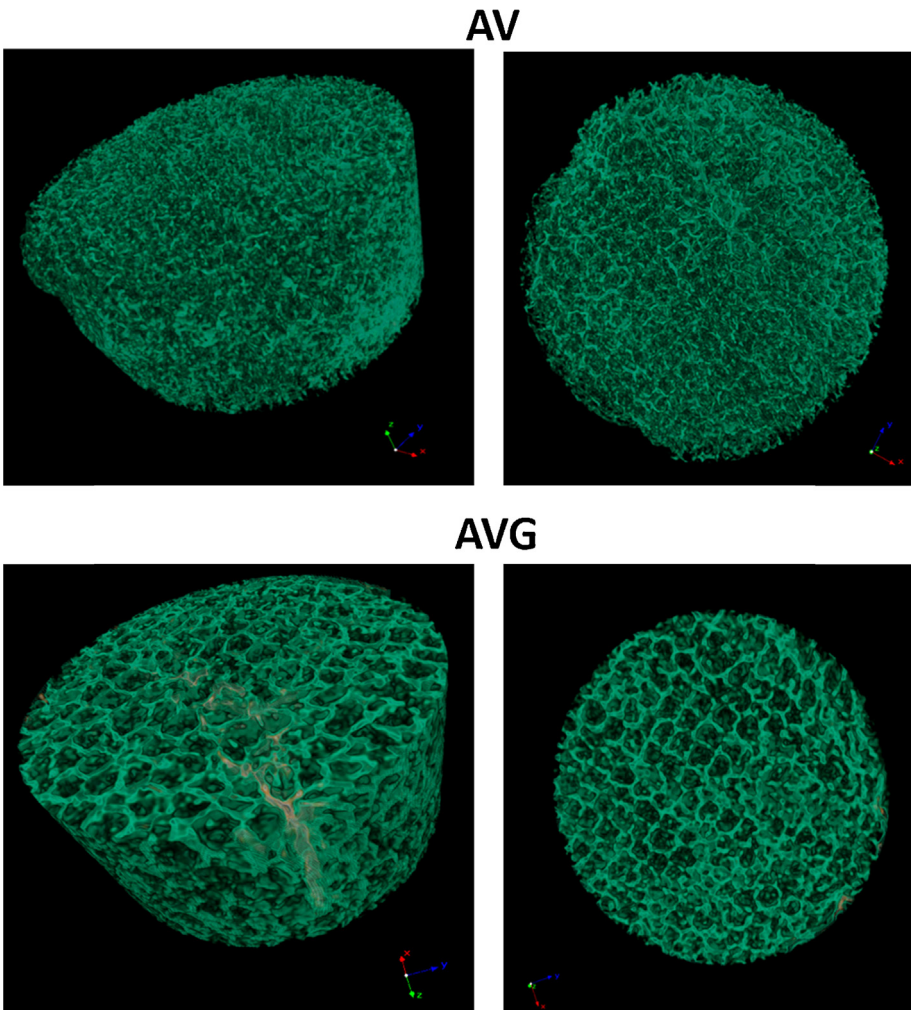

Fig. 2. 3D image reconstructions of the sponges (AV and AVG) obtained by μ-CT seen on a lateral and top views.

Table 1
Values of porosity and interconnectivity for AV and AVG sponges.

Sample	AV	AVG
Porosity (%)	76.9 ± 4.6	72.3 ± 9.4
Interconnectivity (%)	79.3 ± 10.1	74.6 ± 4.9

Micro-CT analysis was used to obtain quantitative information of the 3D architecture of the aloe-based sponges. The 3D micro-CT images, obtained from digital geometry processing from a series of two-dimensional X-ray images, are illustrated in Fig. 2. From the 3D AV image we observed a close pore structure as compared to AVG. The values of interconnectivity (Table 1) indicated that all structures have interconnected pores, which may help homogeneous cell distribution and transferring of nutrients effectively, as well as to facilitate cell growth within the 3D porous structures (Madihally & Matthew, 1999). The pore size distribution of the samples (Fig. 3) presented values ranging between 50 and 300 μm. The AV sponges have also a wide pore size distribution, while AVG sponges have a narrow pore size distribution. In one hand, AV gel is a nature made sponge, possessing high values of porosity (76.9 ± 4.6%) and interconnectivity (79.3 ± 10.1%) that could be adequate for regenerative medicine approaches. On the other hand, the morphological features of AV sponges were affected by the GG coating, where the AVG sponges have values of porosity and interconnectivity lower than AV (Table 1).

4.2. Swelling and stability behavior

Depending on the site of implantation, the biomaterials are subjected to different pH environments, which could affect their degradation, mechanical and swelling properties. To determine how the pH affects the swelling behavior, AVG sponges were immersed in different buffer solutions at pH 5, 7.4 and 10. AVG

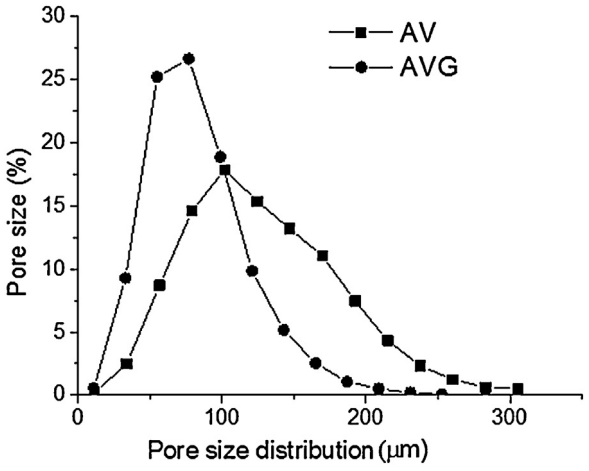

Fig. 3. Porosity distribution of AV and AVG.

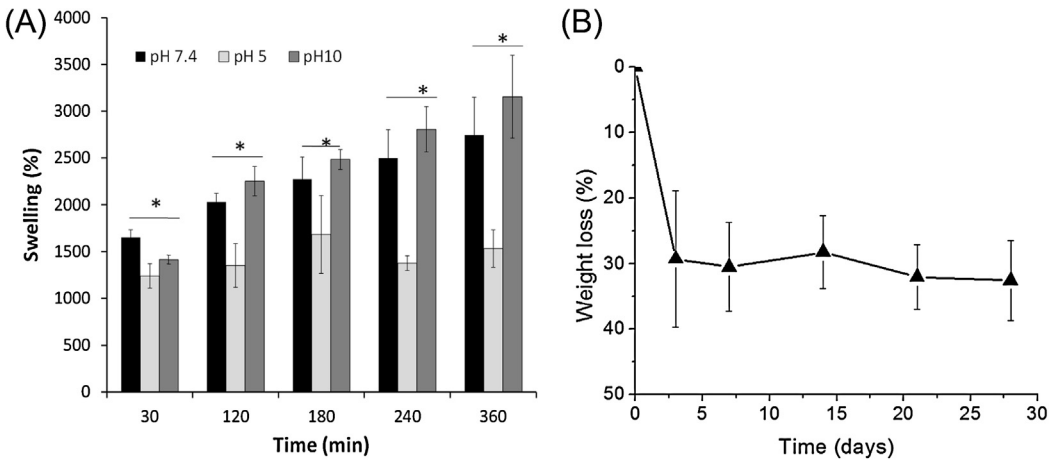

Fig. 4. Swelling profiles of AVG sponges in different buffer pH's (A) and weight loss (B) in function of immersion time on PBS at 37 °C. Data represent the mean ± standard deviation ($p < 0.05$, two-way ANOVA).

samples immersed at pH 10 and pH 7.4 showed an appreciable swelling compared to the ones immersed at pH 5. Statistical differences ($p < 0.05$) were observed for pH 5 in relation to pH 10 and pH 7.4 along the swelling time. The AV gel is rich in hydroscopic polysaccharides such as acemannan (Hamman, 2008), which together with the acid resistance of the coating layer of GG at low pH (Picone & Cunha, 2011), could explain the greater swelling in high pH and its decreasing at pH 5. GG is able to form gels through an ionotropic gelation mechanism (Oliveira, Santos, et al., 2010); where an increase in pH leads to the formation of a reduced number of junction regions (Picone & Cunha, 2011). Other studies on *A. vera*/alginate films demonstrated that an increase in AV content implied an increase in the water absorption ability at pH 7.4 (Pereira et al., 2011), as result of the hydrophilicity of AV.

In tissue engineering approaches, the 3D structures are expected to degrade during tissue regeneration. Thus, it is important to characterize the structural stability of AV-based sponges to understand their potential for application in tissue engineering strategies. The structural stability of the AV and AVG sponges was investigated by weight loss after their incubation in PBS up to 30 days in order to simulate physiological conditions. During the assay, the AV sponges gradually dissolved, leading to the formation of gels and loss of their integrity after a few days (data not shown). Compared with AV, AVG sponges showed a delayed disintegration and increased structural stability. This result could be attributed to the presence of the GG coating on AV. In the presence of salt ions, contained in PBS, the number of junction zones in GG is enhanced (Picone & Cunha, 2011). This leads to an increase in the gel rigidity, increasing its stability to aqueous medium. Furthermore, the AVG sponges showed a weight loss of AVG was about 30 wt% along the degradation period (Fig. 4B) that can be related with the soluble compounds.

4.3. Mechanical properties and in vitro release studies

Almost all biological tissues show native viscoelastic properties (Sasaki, 2012). Those are conferred by constituents such as cells, extracellular matrix and proteins. These properties are of uttermost importance for the correct function of the tissue that must be able to stand the physiological load, as well as be capable of dissipating mechanical energy during cyclic solicitation. Upon the implantation of a biomaterial, it is important to make sure that the structure is compatible also with the tissue mechanical properties, so that it allows for correct biomaterial integration. Moreover, it was shown that the mechanical properties of the biomaterials influence the proliferation and even differentiation phenomena of cells (Engler, Sen, Sweeney, & Discher, 2006). In order to investigate possible applications of the AVG sponges in the regenerative medicine field, we assessed the viscoelastic properties of the structures by dynamic mechanical analysis (DMA). Mechanical analysis of the AV sponge was not conducted as the sponge disintegrate along the tests. The AVG sponges developed in this work present a storage modulus (E') – a measure of the material's stiffness – varying from average values of 5 kPa to 10 kPa (Fig. 5A), over a biological relevant frequency range (0.1–10 Hz, respectively). These E' values are in the range of elastic modulus measured for several soft tissues present in the human body. For example, skeletal muscle was reported to have an elastic modulus of 7 kPa (Chino, Akagi, Dohi, Fukashiro, & Takahashi, 2012) and subcutaneous fat tissue a modulus varied from 0.12 kPa to 30 kPa, according to the applied measuring method (Geerligs et al., 2011; Hendriks et al., 2003). Skin tissue in wet state was evaluated in 10 kPa–100 MPa (Derler & Gerhardt, 2012). Although in this case the E' reported value is higher than the one of the biomaterial proposed herein, it was previously shown that the formation of extracellular matrix by cells seeded in biomaterials increase their stiffness (Popa, Caridade, Mano, Reis and Gomes, 2013; Silva-Correia et al., 2013), and the final tissue may show properties similar to the native tissue (Silva-Correia et al., 2013). Soft biological tissues play an important role in the mechanical integrity of the body. Indeed, these tissues transfer loads between bones to ligaments, or between muscles and bones to the tendons (Silva, Mano, & Reis, 2010). They have an important viscous component in their behavior. The loss factor or damping (herein presented as $\tan\delta$) is a measure of the viscoelasticity of materials, as it correlates the elastic and viscous components. Regarding the loss factor of the AVG sponges, the value 0.5 indicates that the material showed a marked viscoelastic behavior (Fig. 5A). Moreover, this value is also close to the loss factors of native tissues, such as skin, which showed in *in vivo* testing a damping of 0.6–0.8 (Sandford, Chen, Hunter, Hillebrand, & Jones, 2013). Moreover, brain tissue showed loss factor values of 0.4–0.6 (Fallenstein, Hulce, & Melvin, 1969). Considering the results obtained in the analysis of the viscoelastic properties of this new biomaterial, we conclude that it may have potential in tissue engineering of soft tissues, as its properties resemble those from the native tissues.

4.4. In vitro release studies

The characteristics of AV-based sponges could be interesting in the construction of a drug delivery system. In this sense, BSA-FTIC

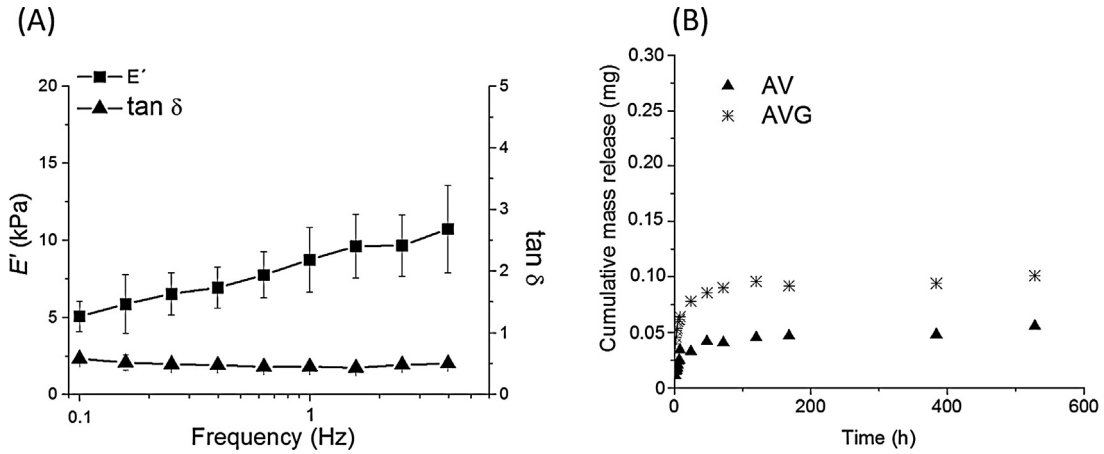

Fig. 5. Evaluation of the viscoelastic properties of AVG sponges by DMA. Frequency scans were performed in the range 0.1–10 Hz under wet conditions at 37 °C (A), and BSA-FTIC release profile of the samples (▲) AV and (*) AVG (B). Data represent the mean ± standard deviation ($p < 0.05$, two-way ANOVA).

was used as a model to analyze their profile release from the AV-based sponges. The release profile of the samples was evaluated in PBS for a time period up to 3 weeks. We hypothesized that the presence of GG coating onto AV (AVG) will act as a barrier to BSA release. In Fig. 5B the release profile of BSA from AV and AVG is shown. It seems that AVG sponges have a faster release than AV, suggesting that the protein was dispersed in the sponge. Although the encapsulated protein on AV could be easily released in the aqueous environment, the findings suggested that the interactions between BSA-FTIC and AV were stronger than AVG, controlling the protein release. Typically, conventional drug delivery systems provide a sharp increase in the concentration of the drug, followed by a rapid decrease in subsequent time periods, until new administration. In controlled delivery systems designed for long term administration the concentration should increase slowly in the beginning of the process, followed by a steady release. The findings obtained for both AV and AVG suggest that a controlled drug release system for therapeutic molecules could be developed from them.

4.5. Cytotoxicity assays

Considering the potential use of these materials in regenerative medicine, a cytotoxicity assessment of both AV and AVG sponges was carried out as a preliminary approach to determine the toxicity potential the sponges. The cell viability values found for AV, AVG extracts and tissue culture polystyrene were 166.9 ± 3.9, 155.8 ± 6.4% and 151.4 ± 8.3%, respectively. The obtained data indicates that both AV and AVG do not exert any cytotoxic effect on L929 cells, which supports the concept that these materials could be used in contact with the body. In fact, the complex composition of AV gel could enhance the wound healing, influencing the wound vascularization and contraction due to the presence of specific active compounds such as beta-sitosterol (Yadav et al., 2012). For instance, AV gel extracts have been shown to stimulate and speed up the production of hyaluronic acid and dermatan sulphate (Chithra, Sajithlal, & Chandrakasan, 1998). Therefore, it is likely that the multiple active phytochemicals present in the AV gel may be required to address different aspects of the wound healing. Further *in vitro* direct cell contact assays will be performed to gain a better understanding of the cellular behavior of AV-based sponges.

5. Conclusions

Versatile 3D sponges were successful developed from native *A. vera* gel using the freeze-drying technique. Both AV-based sponges (AV and AVG) have a heterogeneous porous formation distribution in the matrix, with an interconnected pore structure and good porosity. Moreover, the presence of a coating layer of gellan gum onto *A. vera* matrices improved the stability and mechanical properties of AV sponges. The swelling of AVG was dependent on the pH at which the sponges were prepared. Also, the sponges provided the sustained release of BSA-FTIC, used as a release model for over 3 weeks. Moreover, the low cytotoxicity levels of the sponge extracts are encouraging for further *in vitro* and *in vivo* studies. All findings suggest that the AV-based sponges can be promising candidates for biomedical applications, including in the regenerative medicine field.

Acknowledgments

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