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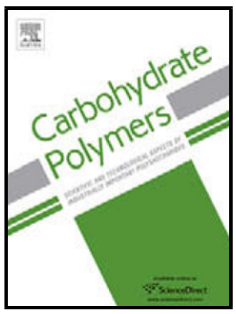

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Effect of crosslinking in chitosan/aloe vera-based membranes for biomedical applications

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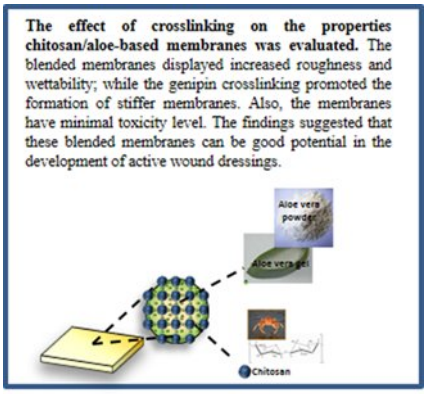
Highlights

- Low crosslinking degree values of the membranes were correlated to blend ratios
- Genipin crosslinking promoted the formation of stiffer membranes
- Blend compositions and crosslinking affected the cellular behavior of the membranes

Abstract

The positive interaction between polysaccharides with active phytochemicals found in medicinal plants may represent a strategy to create active wound dressing materials useful for skin repair. In the present work, blended membranes composed of chitosan (Cht) and Aloe vera gel were prepared through the solvent casting, and were crosslinked with genipin to improve their properties. Topography, swelling, wettability, mechanical properties and *in vitro* cellular response of the membranes were investigated. With the incorporation of aloe vera gel into chitosan solution, the developed chitosan/aloe-based membranes displayed increased roughness and wettability; while the genipin crosslinking promoted the formation of stiffer membranes in comparison to those of the non-modified membranes. Moreover, *in vitro* cell culture studies evidenced that the L929 cells have high cell viability, confirmed by MTS test and calcein-AM staining. The findings suggested that both blend compositions and crosslinking affected the physico-chemical properties and cellular behavior of the developed membranes.

Keywords: Aloe vera; blends; chitosan; crosslinking; genipin.



1. Introduction

The recognition of the pharmaceutical, medical and economical value of medicinal plants is still growing, although this varies widely between countries (Hoareau & Da Silva, 1999). Despite the use of herbal plants over many countries, only a relatively small number of plant species has been studied in a large range of applications. Aloe vera (AV), a tropical plant belonging to the *Liliacea* family (Reynolds & Dweck, 1999), can be found in the formulations of cosmetics, dietary supplements, lotions and beauty products (Eshun & He, 2004). The interest of this plant is associated to its heterogeneous composition. For instance, the AV gel is composed not only by many active compounds but also polysaccharides which express various health benefits (Hamman, 2008). Many studies have been shown that AV has anti-inflammatory, anti-tumor, immodulatory and antibacterial activity (Hamman, 2008; Reynolds & Dweck, 1999). Based on the mentioned properties, current research on AV gel had investigated its use conjugated with synthetic and natural polymers aiming the production of 2D and 3D matrices as *e.g.* hydrogels, nanofibers and films (Inpanya, Faikrua, Ounaroon, Sittichokechaiwut & Viyoch, 2012; Khoshgozaran-Abras, Azizi, Hamidy & Bagheripoor-Fallah, 2012; Park & Nho, 2004; Pereira,

Tojeira, Vaz, Mendes & Bartolo, 2011). In those studies, positive effects on mechanical, biocompatibility and thermal degradation of the resulting matrices were observed due to the incorporation of AV gel.

On the other hand, chitosan, a natural polymer, has been recognized by its intrinsic properties such as biodegradability, solubility in weak acids, cationic nature, anti-bacterial and haemostatic properties (Alves & Mano, 2008; Pillai, Paul & Sharma, 2009). Among the distinct chitosan-based matrices reported in the literature (Guibal, Milot, Eterradossi, Gauffier & Domard, 1999; Muzzarelli, 2009; Reys et al., 2013; Silva et al., 2008) and proposed for biomedical applications, chitosan membranes have been widely investigated for the purpose of wound coverage because of their easy production and long shelf life (Altiok, Altiok & Tihminlioglu, 2010; Silva et al., 2007). Nevertheless, the development of chitosan membranes with desirable properties sometimes requires modification at surface or bulk level by chemical and physical means (Silva, Santos, Coutinho, Mano & Reis, 2005; Silva et al., 2007). Promising findings on wound healing have also achieved using combinations of chitosan and soy protein, alginate or gelatin (Santos et al., 2013; Silva, Mano & Reis, 2010). It has been reported that chitosan-based matrices crosslinked using genipin, an effective natural crosslinking agent, have better stability in aqueous medium and mechanical properties as compared to non-modified materials (Alves & Mano, 2008). Even though the mechanism of reaction of genipin with chitosan is well documented (Muzzarelli, 2009), no information has been found involving reactions of genipin with AV gel. In the present work, blended membranes composed by chitosan and aloe vera gel were produced by solvent casting, and subsequently submitted to crosslinking using genipin. Both native

AV gel and a commercial one (ACTIValoe®) were used in the formulation of the blended membranes in order to study its interactions with chitosan. We hypothesized that these membranes could be used as wound dressings. Then, they will facilitate the the penetration of the AV gel onto the skin, and the membrane may act at different stages of the healing process, while protecting the injury from infection, constituting a more value added material as compared to synthetic ones used for this purpose. Additionally, chitosan will increase the stability of the polysaccharides and/or compounds present in the AV composition (Hamman, 2008), which can in turn keep their natural biological activity. By its turn, the chemical crosslinking on chitosan/aloe-based membranes using genipin will brings the possibility to control the leaching out of AV gel portion from the blended membranes. Herein, issues concerning topography, swelling, wettability, mechanical properties and cellular behavior of the developed membranes were evaluated.

2. Experimental

2.1 *Materials*

Fresh whole aloe vera (*Aloe barbadensis Miller*) leaves obtained in a Portuguese botanic shop, were used as the raw material in all experiments. The studied leaves have between 30 and 40 cm of length, corresponded to 4-year old plants. Aloe vera (Tai-Nin Chow, Williamson, Yates & Goux) commercial powder (Aloe *barbadensis Miller*, ACTIValoe® Aloe Vera Gel Qmatrix 200X) from Aloecorp-Zeus Química Ltda (Portugal) was used as received. Reagent grade medium molecular weight chitosan- Cht (Sigma Aldrich, CAS 9012-76-4) was used, with a deacetylation degree of 76.6%, and molecular weight of 166

kDa, determined by ^{1}H NMR and SEC-MALLS, respectively (Reys et al., 2013). Prior use, chitosan was purified using a re-precipitation method, as described previously (Signini & Filho, 2001). All other chemicals were reagent grade and were used as received.

2.2. *Methods*

2.2.1 *Extraction of the aloe vera gel*

Whole aloe vera leaves were washed with distilled water to remove dirt from the surface. The skin was carefully separated from the parenchyma using a scalpel-shaped knife. The filets were extensively washed with distilled water to remove the exudates from their surfaces. The fillets were homogeneized in a blender and then the homogenized mass was filtered. After that the AV gel was stabilized at 65°C for 15 minutes and stored at 4°C prior uses.

2.2.2. *Preparation of the membranes*

Chitosan flakes were dissolved in an aqueous acetic acid 0.2 M at concentration of 2 wt% (w/v) to obtain a homogeneous solution. The chitosan/aloe vera gel blended films were prepared using two strategies. In the first approach, the extracted AV gel was added to Cht solution at a ratio of 1:2, 1:3 and 2:1 v/v Cht/AV. In the second approach, the AV powder (ACTIValoe®) was added to Cht solution at ratio, 1:2, 1:3 and 2:1 (w/v). Glycerol, a well-known plasticizer, was also added to blended mixture (water/glycerol 2.5% v/v). Then, the blended systems were kept under stirring at 4°C for at least 3 hours. After homogenization, the blended solutions were casted into petri dishes and dried at room temperature for 4 days. Subsequently, the neutralization of the

membranes was performed by soaking them in a NaOH 4%/ethanol 1:1 for 10 minutes, followed by washing with ethanol and then with distilled water until pH 7. For Cht membranes, the neutralization was performed using only NaOH 4% w/v. The identification of the Cht/AV membranes was CA and CAQ, for those prepared using native and commercial AV, respectively.

2.2.2.1. *Determination of aloe vera release*

The aloe release from the membranes (CA and CAQ), during neutralization process, was calculated using a standard curve constructed using freeze-dried AV gel (concentrations ranging from 0.01-1 mg/ml) dissolved in NaOH/ethanol 1:1 (v/v). Then the optical absorbance of the solution was recorded with a spectrophotometer (Bio-Rad SmartSpec 3000, CA) at a wavelength of 400 nm. All tests were performed using six replicates.

2.2.3. *Genipin crosslinking on membranes*

Dried CA and CAQ membranes were crosslinked by immersion in a bath containing genipin 10 mM, previously dissolved in pure ethanol. The crosslinking reaction was performed at room temperature during 30 minutes and then, the membranes were washed with ethanol, followed by distilled water to remove non-reacted genipin. The membranes were dried at room temperature and stored at 4°C until use. The identification of the crosslinked blended membranes was CAG and CAG1, prepared using native and commercial AV gel, respectively. The crosslinking degree of each test group of samples (non- and crosslinked membranes) was determined by the use of the ninhydrin assay (Eick, Good & Neumann, 1975). In the ninhydrin assay, the sample was weighed (3 mg) and heated with a ninhydrin solution (2 wt % v/v) at

100°C for 20 min. Then the optical absorbance of the solution was recorded with a spectrophotometer (Bio-Rad SmartSpec 3000, CA) at a wavelength of 570 nm. Glycine solutions of various known concentrations were used as standards, and both CA and CAQ membranes that were prepared without genipin were used as control materials. After the sample was heated with ninhydrin, the number of free amino groups in the test sample was proportional to the optical absorbance of the solution. Each sample was made in triplicate. Then, the degree of cross-linking of the samples was calculated (Equation 1). Fresh and fixed are the mole fractions of free NH_2 remaining in non-crosslinked and crosslinked samples, respectively.

$$Degree\ of\ croslinking = \left[\frac{(NH\ reactive\ amine)\ fresh - (NH\ reactive\ amine)fixed}{(NH\ reactive\ amine)fresh}\right] \times 100 \qquad \text{Equation 1}$$

2.3 **Characterization**

2.3.1. *Atomic force microscopy (AFM)*

The samples were measured on at least three spots using TappingMode™ with a MultiMode connected to a NanoScope, both supplied from Veeco, USA, with non-contacting silicon nanoprobes (ca 300kHz, setpoint 2-3V) from Nanosensors, Switzerland. All images (10 μm wide) were fitted to a plane using the 3rd degree flatten procedure included in the NanoScope software version 4.43r8. The surface roughness was calculated as Sq (root mean square from average flat surface) and Sa (average absolute distance from average flat surface). The values are presented as mean (standard deviation).

2.3.2. *Swelling*

Swelling behavior of the membranes was performed by immersing the membranes in phosphate buffer solution (PBS) at 37°C. The swollen sample weights were measured after removing excess surface water by gently tapping the surface with filter paper. Water uptake was determined from the swollen state, w_s and the final dried weight w_f using Eq. (2). Each experiment was repeated three times, and the average value was considered to be the water uptake value.

$$Swelling\ (\%) = \left[\frac{(Ws - Wd)}{Wd}\right] x\ 100$$

Equation 2

2.3.3. *Contact angle measurements*

The surface properties of the membranes were also investigated by means of static contact angle measurements using the sessile drop method with glycerol (polar) and diiodomethane (non-polar) (OCA equipment, Germany and SCA-20 software). Six measurements were carried out for each sample. The presented data (Table1) corresponds to the average of six measurements. The surface energy was calculated using the Owens, Wendt, Rabel and Kaelble (OWRK) equation (Owens & Wendt, 1969).

2.3.4. *Dynamical mechanical analysis*

The viscoelastic measurements were performed using a TRITEC8000B dynamic mechanical analyzer (Triton Technology, UK) equipped with the tensile mode. The measurements were carried out at 37°C. The distance between the damps was 10 mm and the samples were cut with 6 mm width (measured

accurately for each sample). The membranes were always analyzed whilst immersed in a liquid bath placed in a Teflon reservoir. The samples were clamped in the DMA apparatus and immersed in PBS solution. 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). A minimum of three samples were used for each condition.

2.3.5. *Screening cellular behavior*

In vitro tests were performed using a cell suspension of the mouse fibroblast-like cell line L929 at a passage P16 (L929 cells; European Collection of Cell Cultures-ECACC, UJ). All membranes were sterilized under an ethylene oxide atmosphere. Cells were seeded in the samples with a density of 4×10^4 cell/cm^2 nourished with Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic. Cultures were incubated at 37°C in a humidified 95% air and 5% CO_2 atmosphere for 1, 3, and 7 days. Triplicates were used for each time point and for each different test. After each time point, the medium was removed and samples were washed with PBS and processed for alamar blue, DNA quantification and calcein-AM staining. AlamarBlueTM assay was performed to assess the metabolic activity of L929 cells in contact with the membranes along the culture. For this assay, an alamar blue TM solution was prepared and added into medium, followed by a 4 hours incubation period at 37°C. Finally, the optical density was read at 570 and 600 nm on a multiwell microplate reader (Synergy HT, Bio-Tek Instruments). All tests were performed using 6 replicates. L929 cell proliferation in the both Cht and Cht/AV-based membranes was determined

using a fluorimetric double strand DNA (dsDNA) quantification kit (PicoGreen®, Molecular Probes, Invitrogen, UK). Herein, samples collected at 1, 3 and 7 days were transferred into 1.5 ml microtubes containing 1 ml of ultra-pure water. Cell-membrane constructs were incubated for 1 h at 37 °C in a water-bath and then stored in a -80°C freezer until testing. Prior to dsDNA quantification, constructs were thawed and sonicated for 15 min. Samples and standards (ranging between 0 and 2 mg/ml) were prepared and mixed with a PicoGreen solution in a 200:1 ratio, and placed on an white 96 well plate. Triplicates were made for each sample or standard. The plate was incubated for 10 min in the dark and fluorescence was measured on a microplate reader (BioTek, USA) using an excitation and emission wavelengths of 485/20 nm and 528/20 nm, respectively. A standard curve was created and sample dsDNA values were read off from the standard curve graph. For the fluorescent images, calcein AM was used to stain the cells. A 2μL amount of calcein AM and 1 mL of D-MEM culture medium without phenol red or FBS were added to each sample, followed by 10 min of incubation at 37°C. Live cells stained green due to enzymatic conversion of the nonfluorescent cell-permeant calcein AM to fluorescent calcein. Fluorescent cells were visualized with the corresponding filters under an inverted microscope (Imager-Z1M).

Statistical analysis

All quantitative experiments are run in triplicate and results are expressed as mean ± standard deviation for n=3. Statistical analysis of the data was conducted using two-way ANOVA with Bonferroni´s post-test by using 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.

3. Results and discussion

3.1. General aspects

Chitosan/aloe vera (Cht/AV)-based membranes were successfully prepared. Two types of Cht/AV membranes were produced: **CA**- membranes prepared using AV gel obtained directly from the leaves of the plant; and **CAQ**-membranes produced using AV gel commercial powder. The membranes have a yellowish colour (see Figure 1) with a thickness in a range of 129 to 425 μm.

Figure 1

Glycerol, added to all formulations, acted as a plasticizer and it had an important role in the improvement of the chain flexibility and resistance of the membranes: without it, the blended membranes were too fragile for handling. The effect of glycerol as plasticizer agent has been observed in other studies involving chitosan membranes (Larena & Cáceres, 2004). Among all the prepared formulations, those prepared with a ratio of 2:1 (CA) and 1:2 (CAQ) showed better mechanical stability. Then, the characterization continued only with the membranes prepared using these two ratios.

During the neutralization process on all Cht/AV-based membranes using NaOH solution, a considerable leaching out of AV was observed (data not shown). To avoid the swelling of the membrane, a NaOH/ethanol (1:1) solution was used instead only aqueous NaOH solution. Caridade *et al* (Caridade, da Silva, Reis & Mano, 2009) observed that the swelling of chitosan membranes can also be controlled varying the composition of water/ethanol solution. The AV release

calculated on the washing solutions along the neutralization process was around 2% (w/v) for CA and 10% (w/v) for CAQ. These results suggested that the interaction between Cht and native AV gel was better than with commercial AV gel. Moreover, chemical crosslinking on blended membranes was investigated as an approach to increase the stability of aloe fraction in the membrane, which will imply in an improvement of their physical properties. Both crosslinked membranes (CAG and CAG1) showed good handling properties and, the crosslinking degree values found were 1 and 15% for CAG and CAG1, respectively. It is known that genipin crosslinking reactions occur spontaneously with the amino groups of chitosan (Butler, Ng & Pudney, 2003) and amino acids such as lysine and arginine present in the proteins (Sung, Huang, Huang, Tsai & Chiu, 1998). In the Cht/AV-based membranes, chitosan has a high amount of amino groups (DD 76.6%), while AV contains amino acid in a varying percentage (Hamman, 2008). Then, the crosslinking values may be associated with different interactions of genipin with the blend components. Previous studies (Mi, 2005; Silva et al., 2008) on crosslinking of genipin on chitosan blends suggested that the speed of the reactions as well as the crosslinking degree may be associated to blend ratio.

3.2. Surface Topography

Changes on the surface topography at a nanometer level of the membranes are showed in Figure 2. From AFM data, an increasing surface roughness was observed in the blended membranes, from 3.8 ± 0.5 (Cht), 5.4± 1.0 (CA) to 45.7 ± 11 nm (CAQ). The presence of AV could promoted an arrangement of the macromolecules into the blended system that may have contributed to these findings. Furthermore, the crosslinked membranes presented lower roughness

values as compared to non-crosslinked ones, from 4.4 ± 2.4 (CAG) to 34.9 ±1.3 (CAG1) nm. In these cases, the possible AV leaching out during crosslinking reaction may be a reason for these results.

Figure 2

3.3 Hydrophilic/hydrophobic balance and swelling behaviour

The measured contact angle and the calculated surface energy of the studied membranes are summarized in Table 1. By findings, the incorporation of AV promoted an increase in the surface hydrophilicity, while the genipin crosslinking promoted the formation of hydrophobic network membranes. In fact, the reaction of genipin with primary amino groups (Cht) and amino acids may result in a decline in the $–NH_2$ group content and the membrane becomes more hydrophobic. It may assumed that the observed variations in the contact angle of the blended membranes probably occurred due to the changes on the surface roughness and/or a re-structural arrangement of the blend components. Further the surface energy was calculated using the Owens, Wendt, Rabel and Kaeble (OWRK) method (Owens & Wendt, 1969). As shown in Table 1, the total surface energy was higher for modified membranes than for the Cht, which suggested the generation of more reactive surfaces. Also, both the incorporation of AV and genipin crosslinking resulted in a variation of the polar component in the modified membranes. It could be possible that the different polar groups present in the AV gel (Hamman, 2008) affected the final blend composition (CA and CAQ); which in turn may affect the surface energy and its components. Furthermore, the chemical crosslinking will increase the interaction between Cht and AV chains become the membranes more

hydrophobic, and then decreasing the polar component fraction. The contact angle data on CAG and CAG1 help to support these hypothesis.

Table 1

Figure 3 displays the swelling behavior of the membranes in PBS up to 24 hours. All the membranes revealed to be stable in the PBS solution, but presented different water uptake profiles. It is observed that the water uptake of CA enhanced over the Cht itself (Figure 3a). Statistical differences ($p<0.05$) were found between Cht and CA profiles up to 24 hours. This result should be a consequence of the increase of hydrophilic groups in the blended system due to the addition of AV. A similar behavior was reported when AV was conjugated with bacterial cellulose (Saibuatong & Phisalaphong, 2010). On the other hand, the swelling behavior of the crosslinked membranes has also differing profiles, where an increasing of the water uptake on CAG and a decreasing on CAG1 were observed as compared to Cht (Figures 3a-b). Statistical differences ($p<0.05$) were detected between the pairs CA/CAQ, and Cht/CAG. Despite the crosslinking degree of CAG1 (15%), perhaps many hydrophilic groups present in this formulation were not crosslinked, which explain its behavior. Also, the differences observed between CA and CAG can be associated to its hydrophobic character showed in CAG by contact angle measurements (Table 1).

Figure 3

Mechanical and viscoelastic properties

DMA experiments were performed in a hydrated environment at 37ºC to assess how membranes behave in more realistic conditions. Figure 4 presents the viscoelastic behavior of the membranes, where the storage (elastic) modulus, *E*', and the loss factor, tan δ, were determined. The mechanical analysis of membranes made of AV gel alone was not conducted because its film was too fragile for handling. As shown in Figure 4a and 4b, *E'* of all membranes tend to increase with increasing frequency. From figure 4a, the results revealed that both CA and CAG were stiffer than Cht membranes alone, exhibiting the highest *E*' values. In figure 4b, the same tendency was observed for Cht and CAQ. The crosslinking of the membranes increased significantly their stiffness (see figure 4a and 4b). These findings suggest that both the addition of AV and genipin crosslinking contributed positively, enhancing the interaction between the blend components and, consequently, for membranes with a rigid network and higher stiffness. Pereira et al (Pereira, Tojeira, Vaz, Mendes & Bartolo, 2011) also reported the improvement of the mechanical properties of AV/alginate films. Figures 4c and 4d represent the variation of the tan δ along the frequency. The loss factor is the ratio of the amount of energy dissipated by viscous mechanisms relative to energy stored in the elastic component providing information about the damping properties of the material (Mano, 2008). For all formulations, tan δ decrease with the increase of frequency indicating that the materials became less viscous and more elastic. Moreover, from figure 4c, it was observed that the tan δ exhibited higher values for CA and CAG than Cht indicating that such materials possess higher capacity to dissipate energy. A similar tendency was verified, in figure 4d, for CAQ and CAG1 membranes. Statistical differences ($p<0.05$) were found between the

crosslinked (CAG and CAG1) and the uncrosslinked (CA and CAQ) membranes).

Figure 4

Cellular behavior

The developed blended membranes varied not only by their mechanical properties, but also by surface properties. These factors can influence their cellular response and, consequently will determine their applications. Thus, a preliminary cellular screening was performed using direct contact tests with L929 fibroblast-like cells. The alamar blue results (figure 5A) revealed that the L929 cells were metabolically active when cultured on the developed membranes. Interestingly, the metabolic activity of cells on both CA and CAG was superior to the observed in Cht membranes. Significant differences on cell metabolic activity ($p<0.05$) was found for Cht and CAQ along the studied culture time. The DNA results (Figure 5B) showed that L929 are able to proliferate after 3 days, excepted for CAG. Statistical differences ($p< 0.05$) were found in the cell proliferation on day 7 between all non- and cross-linked blended membranes. Some reports described the stimulatory effect of aloe extract and their components on cell proliferation; however, the identity of the substances responsible for influencing cell proliferation is currently unknown (Chang, Chen & Feng, 2011). There may be a synergistic effect between polysaccharides, proteins and other low-molecular compounds that make part of the AV gel composition that helps on this behavior. In contrast to the positive effects observed previously, both CAQ and CAG1 have differing effects, where cell proliferation was not observed for CAG1. The addition of certain substances in

the commercial AV, during its processing, can affect negatively the bioactivity of the gel and, consequently its cell proliferation (Reynolds & Dweck, 1999). Additionally, the crosslinking of genipin on AV gel can be blocked their active molecules, which help to explain their cellular behavior. Furthermore, the calcein-AM results (Figure 6) are in agreement with the findings obtained from the alamar blue assay (Figure 5a) and DNA quantification (Figure 5b), suggesting that cells are viable on the membrane surface. Nevertheless, the cell behavior was different depending on the membrane composition.

Figure 5

On the Cht membrane (Figure 6a) membranes, the fibroblasts exhibited a round morphology with cell agglomeration throughout the study period. In the CA (figure 6b), the cells are spreading on the surface exhibiting a spindle-like shape behavior that suggested a good adherence to this surface. Moreover, the CAQ (Figure 6d) and those submitted to genipin crosslinking (CAG- Figure 6c and CAG1- Figure 6e) did not show a good cell viability as compared to CA (Figure 6b). According to the literature (Zhu, Chian, Chan-Park & Lee, 2005), unmodified Cht membranes would not be able to promote fibroblast cell attachment and proliferation, probably as result of the monopolar basic nature of Cht, which does not interact well with the bipolar extracellular matrix proteins present in the bovine serum proteins of the culture medium. An improvement of cell behavior onto chitosan-based membranes have been evidenced after incorporation of others polysaccharides or proteins to chitosan (Silva, Santos, Coutinho, Mano & Reis, 2005; Silva, Mano & Reis, 2010). In this present study, the conjugation with AV gel, mainly the native one, with Cht contributed to a better adhesion of fibroblasts on surface of the developed blended membranes.

The cellular behavior observed on Cht/AV-based membranes can be also influenced by alterations of their physical features such as the surface energy, topography and stiffness, as reported in other studies (Chu, Chen, Wang & Huang, 2002).

Figure 6

4. Conclusions

Non- and crosslinked chitosan/aloe vera-based membranes were successfully produced. The properties of the developed membranes were mainly influenced by chitosan/aloe vera ratio and genipin crosslinking. As expected the genipin crosslinking enhanced the stability and mechanical properties of the blended membranes. Furthermore, i*n vitro* screening cellular behavior revealed that all developed blended membranes have differences on cell behavior which were associated to aloe sources (commercial and native one) and their physical features (surface energy, topography and stiffness). Thus, the use of aloe vera gel native combined with chitosan appears to have potential in the development of membrane as active wound dressings.

Acknowledgments

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Figure Captions

Figure 1. Photographs of the blended membranes; (a) CA and (b) CAQ.

Figure 2. AFM 3D images of the developed membranes.

Figure 3. Water uptake of the developed membranes; (a) Cht, CA, CAG and (b) Cht, CAQ, CAG1 as function of immersion time on a PBS. Data represent the mean ± standard deviation ($p < 0.05$, two-way ANOVA).

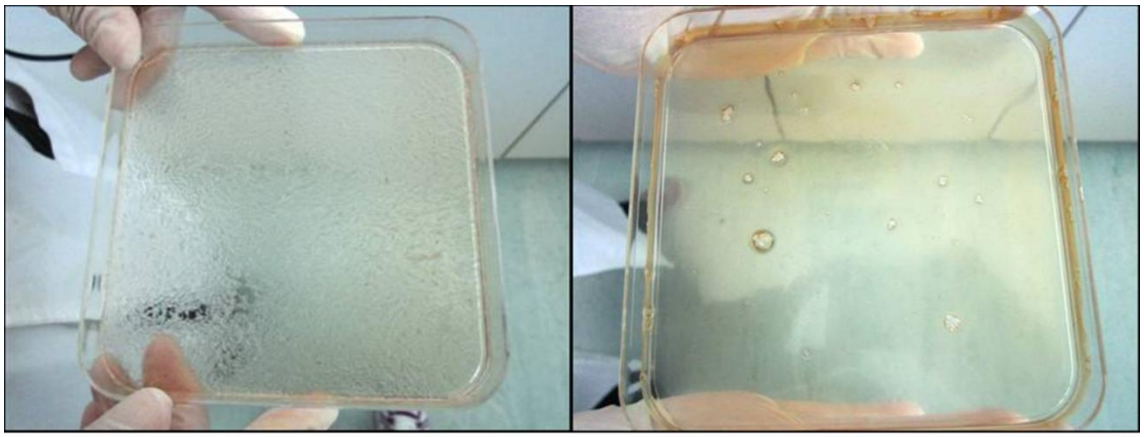
Figure 4. Evaluation of the viscoelastic properties of chitosan/aloe-based membranes using DMA. Frequency scans were performed in a range of 0.1 to 10 Hz in wet conditions at 37ºC. (a): E' of Cht, CA and CAG membranes; (b): E' of Cht, CAQ and CAG1 membranes; (c): tan δ of Cht, CA and CAG membranes and (d): tan δ of Cht, CAQ and CAG1 membranes.

Figure 5. (A) Alamar blue results of L929 cells on Cht/AV-based membranes as a function of culture time. The values were normalized to cell viability on TCPS, which was used as a positive control of cell viability; (B) dsDNA content of L929 cells on Cht/AV-based membranes as a function of culture time. Data represent the mean ± standard deviation ($p < 0.05$, two-way ANOVA).

Figure 6. Fluorescent images of L929 cells stained with calcein- AM after 3 days of culture on membranes: chitosan (a), CA (b), CAG (c), CAQ (d) and CAG1 (e).

Table 1. Contact angles, dispersive (γ_d) and polar (γ_p) components of the prepared membranes calculated by the Owens – Wendt equation.

Membrane	**$\theta_{glycerol}$ (°)**	**γ_d (mN.m^{-1})**	**γ_p(mN.m^{-1})**	**γ (mN.m^{-1})**
Cht	88.4 ± 0.9	13.3 ± 0.1	6.0 ± 0.1	19.3 ± 0.02
CA	86.4 ± 2.8	21.7 ± 0.1	3.1 ± 0.01	24.8 ± 0.01
CAG	94.4 ± 2.2	23.5 ± 0.2	1.1 ± 0.03	24.6 ± 0.02
CAQ	82.8 ±2.9	24.8±0.1	3.7±0.01	28.5 ±0.1
CAG1	90.9± 2.7	32.3±0.1	0.5±0.01	32.8±0.1

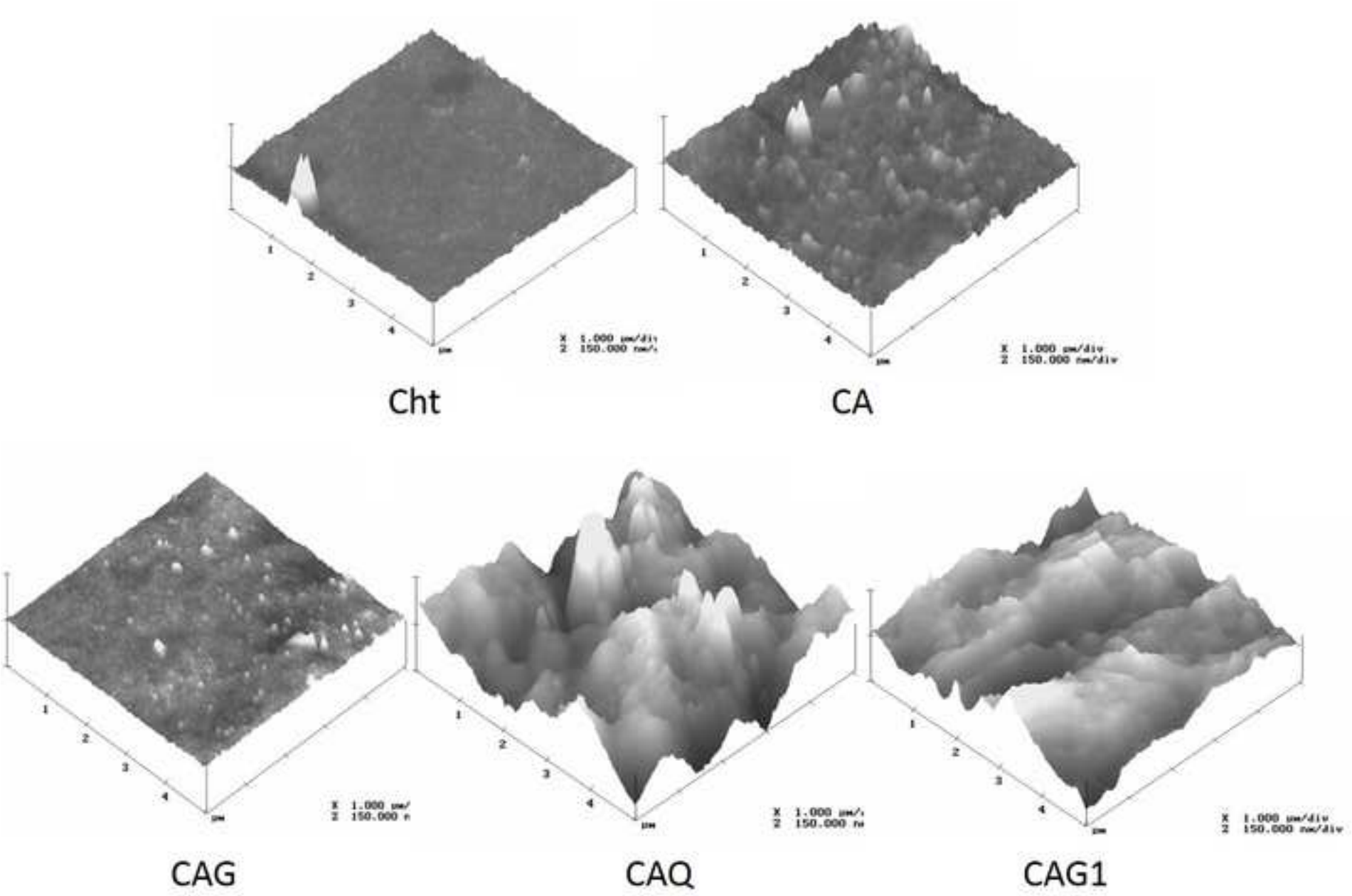


(a) (b)

Figure 1. Silva SS et al.

Figure 2

Click here to download high resolution image



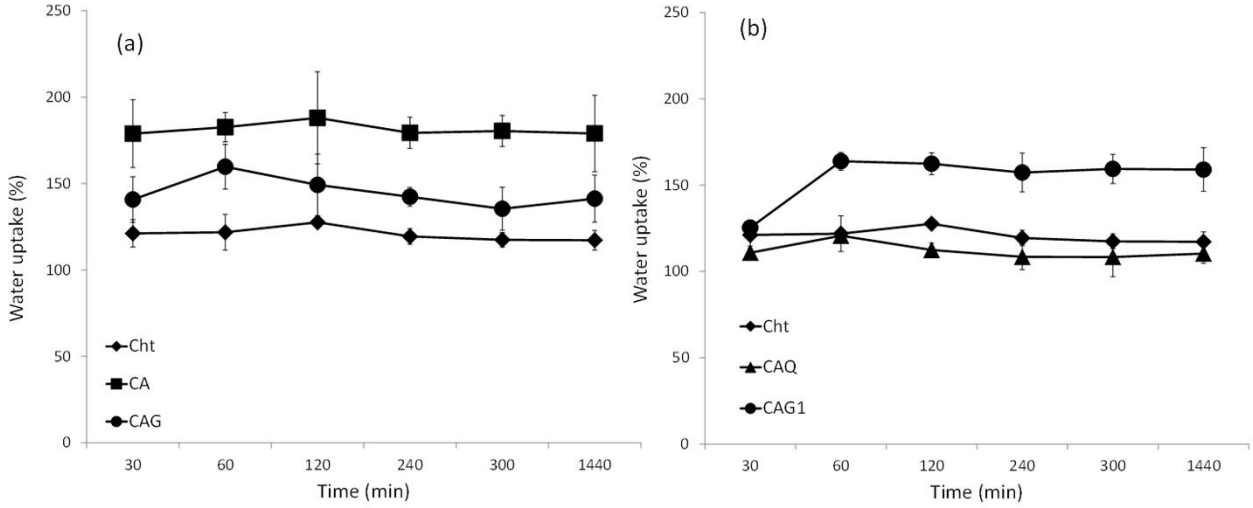


Figure 3. Silva SS et al.

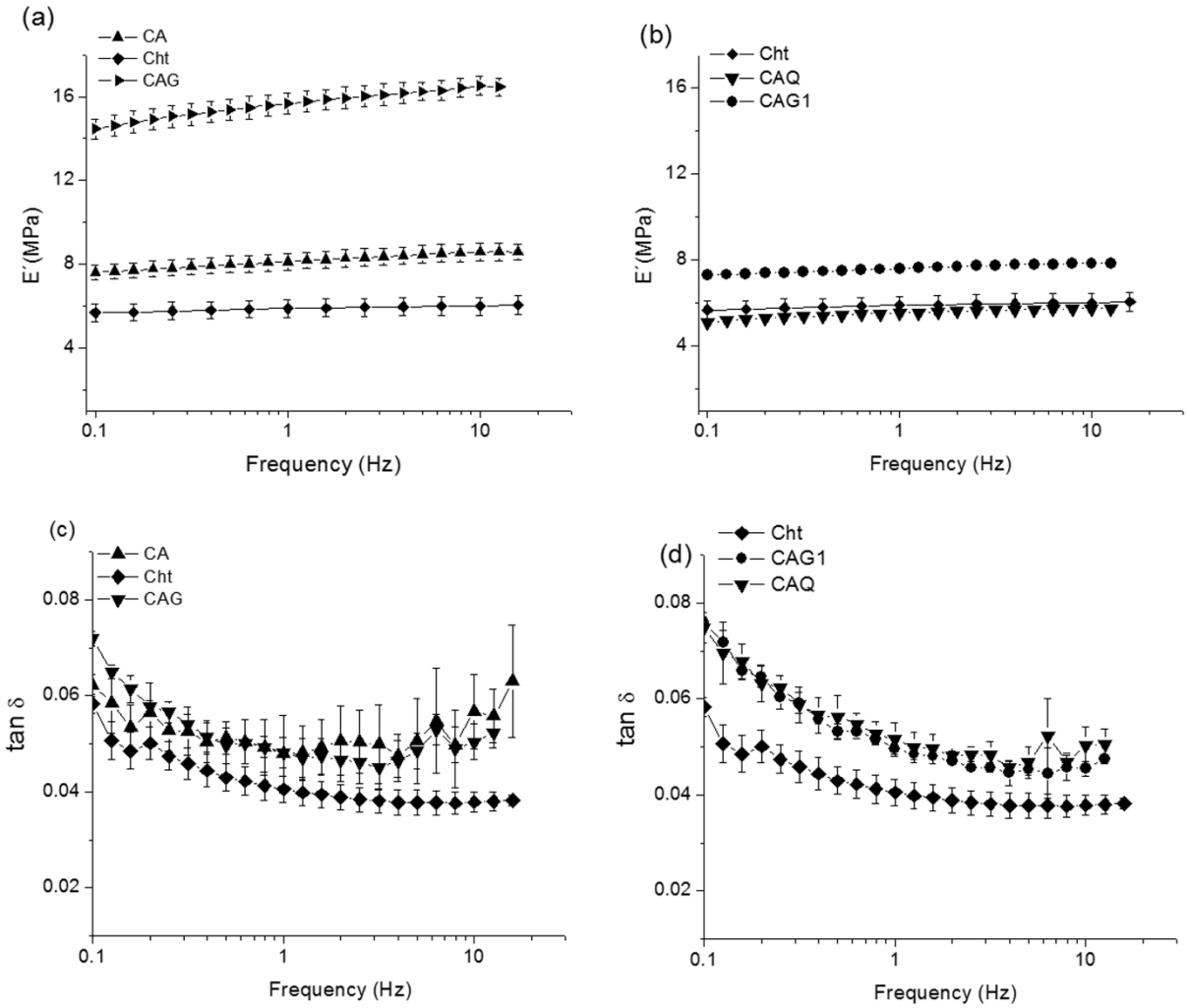


Figure 4. Silva SS et al.

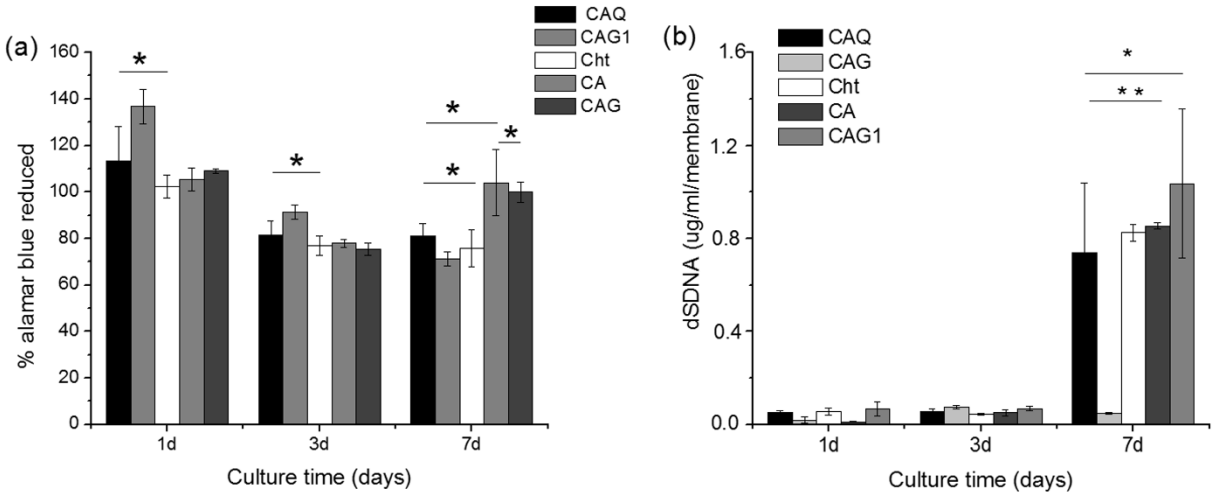


Figure 5. Silva SS et al.

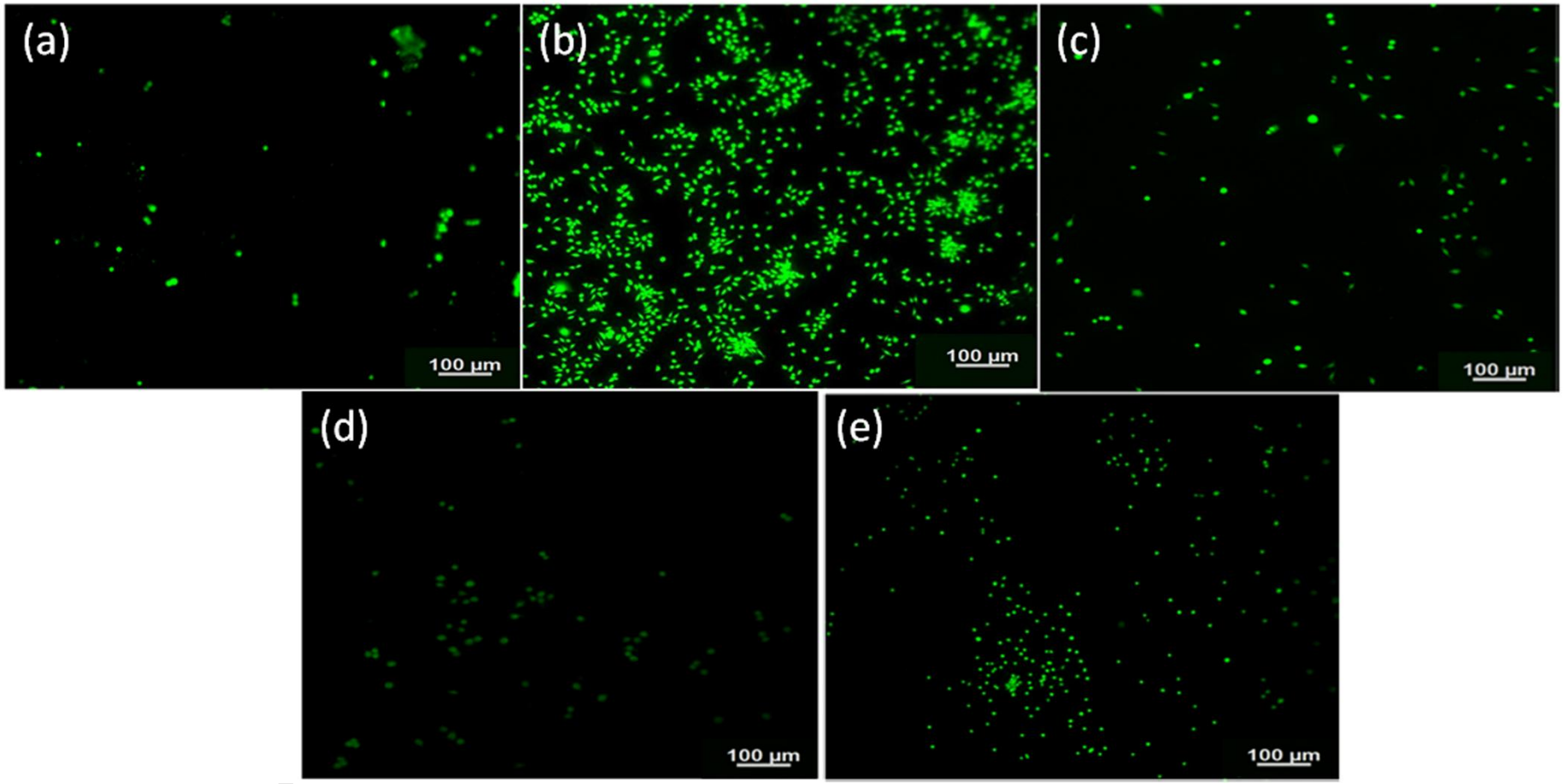


Figure 6. Silva SS et al.