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STATISTICAL APPROACH OF THE PHYSICALCHEMICAL INVESTIGATION OF CHITOSAN/COLLAGEN BIOMATRIX CROSSLINKED WITH GRADIENT OF GENIPIN FOR TISSUE ENGINEERING

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ABSTRACT

Introduction: More than 60 million people in the United States have had an increase in their life expectancy in recent years because of artificial tissue and organ therapy, with every five people over 65 years old. In Brazil, this figure was higher. Thus, tissue engineering contemplates numerous advantages that meet the needs of the injured tissue or organ for the regeneration process.

Objective: to investigate the physicochemical properties of chitosan/collagen cross linked with a gradient of genipin for tissue engineering.

Methods: Chitosan/collagen biomatrices were prepared and crosslinked with a gradient of genipin for swelling, degradation and cross linking degree investigations, with statistical approach.

Results: 0.75 % v/v genomic composite biomatrix indicated to present the best physicochemical characteristics for future clinical studies.

Conclusion: With the increase of the degree of interlocking with genipin, the biomatrices became more rigid, reducing the degree of swelling and of free amino groups. Thus, through the present investigation, the chitosan/collagen biomatrix with 0.75 % v/v genipin provided the best physicochemical properties for future cell and clinical studies.

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INTRODUCTION

More than 60 million people in the United States have had an increase in their life expectancy due to the therapy of artificial tissues and organs, and every five people over 65 years of age have benefited from tissues and organs generated "in vitro" (Muzzarelli *et al.,* 2016; Varoni *et al.,* 2017; Abbott, 2012). In Brazil, this figure was higher (Zotarelli Filho *et al.,* 2015). Thus, it is imperative to develop new strategies to meet the demand with the development of biomaterials, whose main purpose is the regeneration of tissues and organs and the amortization of costs (Muzzarelli *et al.,* 2016; Alsarra, 2009;

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Baldwin and Kiick, 2010). Thus, tissue engineering contemplates numerous advantages that meet the needs of injured tissue or organ for the regeneration process (Yang *et al.,* 2017; Beppu *et al.,* 1999). For this, the understanding of chemical, physical and biological processes is necessary both biological material and the biological niche of the host (Varoni *et al.,* 2017; Ko *et al.,* 2010). The cross-referencing of compatible information between microenvironments allows cell recognition and signaling cascades for neovascularization (Boccafoschi *et al.,* 2005). Another advantage is the minimally invasive surgical intervention, which allows the use of surgical techniques that are faster and cause less risk to the patient (Langer and Vacanti, 1999). For this, in the present study, chitosan-collagen-genipin biomatrices were used, because chitosan presents low molar mass with degrees of deacetylation greater than 0.4 are easily soluble in acidic solvents (Yang *et al.,* 2017; Alsarra, 2009). The physico-chemical behavior of chitosan in aqueous solutions is highly dependent on pH and degree of deacetylation and has been the target of a large number of studies (Yang *et al.,* 2017; Alsarra, 2009). Thus, the degree of deacetylation (GD) is an important criterion in the activity of chitosan in the process of tissue regeneration. This was analyzed with the in vitro growth of cultures of human keratinocytes and fibroblasts treated with chitosan solutions with different degrees of deacetylation (Chan and King, 2009). On the other hand, collagen is an important biopolymer in tissue engineering and is present in several commercial products used in dermatology and aesthetics (Bet *et al.,* 1997). Thus, because of its physicochemical and biological properties, collagen can be processed in different geometric forms without losing its intrinsic properties (Panopoulos *et al.,* 2012).

It can undergo chemical changes by cross-linking and hydrolysis for use as matrices both in their pure form, as in collagen gels, or in the form of blends with chitosan and other biopolymers. The mixture of the two biopolymers reconciles the biocompatibility of collagen with the adhesion forces of chitosan (Domard and Taravel, 1995). Collagen is bioadhesive by specific arginine-glycine-aspartate (RGD) sites (Zotarelli Filho *et al.,* 2013). The RGD group promotes cell adhesion through binding to integrin receptors, thus promoting cell growth and differentiation (Zotarelli Filho *et al.,* 2013). In addition, genipin is a hydrophilic organic compound that had its structure discovered in 1960 and is extracted from geniposide (origin: Gardenia fruit) (Jin *et al.,* 2004). Although biocompatibility has not been tested in humans, it has been shown that genipin is non-cytotoxic "in vitro" and that it is biocompatible in rats Sung *et al.* (1999). verified that genipin was 10,000 times less cytotoxic than glutaraldehyde, which justifies its use as a cross-linking agent in biomaterials without causing chronic inflammatory problems. Therefore, in this scenario of technological advances in regenerative medicine and human tissue engineering, the present study aimed to investigate statistically the physicochemical properties of chitosan / collagen crosslinked biomatrix with genipin gradient for tissue engineering.

MATERIALS AND METHODS

Materials

Chitosan, collagen and genipin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen type 1 bovine from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and other reagents also were purchased from Sigma-Aldrich (St. Louis, MO, USA). Device Microplate Spectrophotometer (BioTek® Instruments, USA).

Protocols

Preparation of the chitosan-collagen-genipin biomatrix: Based on the method described by Baldwin; Kiick (2010), the previously characterized chitosan (Mw = 115 kDa and degree of deacetylation of 85.25 %) was dissolved in 10.0 mL of 2.5 % v/v acetic acid solution for 24 hours at room temperature. The collagen was dissolved. The chitosan-collagen biomatrix was prepared by mixing the two solutions under stirring for 96 h. The solutions were transferred to a 96-well plate, with a volume of 170.0 µL, in a ratio of 1: 1 v/v. Finally, the biomatrix was crosslinked obeying the concentration in ascending order of genipin of 0.10 %, 0.25 %, 0.50 %, 0.75 % and 1.00 % v/v. The mixture was then frozen in liquid nitrogen (-196.0 ° C) and then lyophilized.

Determination of the degree of swelling (DS) of the biomatrizes: The matrices were weighed when dry and swollen with alpha MEM cell culture medium (pH = 7.4) to determine the degree of swelling, with n = 10 and the measured data were studied and analyzed in the Minitab 17 statistical program. The amount of blood in absorbed alpha MEM was calculated by equation 1:

$$DS\ (\%) = (m_w - m_d) / m_w\ (x\ 100) \qquad \text{(Equation 1)}$$

Where : m_w = wet mass
m_d = dry mass

Study of the "in vitro" degradation of the biomatrices with blood sample in ALFA MEM culture medium: For the determination of the degradation index (DI) the samples in replicates with initial mass, M_i, were oven dried at 40 ± 2oC and weighed after weight stabilization (M_{id}). After weighing the samples were placed in containers with blood solution in ALFA MEM culture medium obeying the relation between the surface area and the volume of solution = 0,1 cm -1 (27,28). Samples were maintained at (37 ± 1 ^{0}C) in a water bath. Then, they were removed from the container and dried in an oven at (40 ± 2 ^{0}C), after reaching weight stabilization it was recorded. The DI was obtained according to equation 1, where M_{id} is the initial dry mass and M_{fd} is the mass of the sample after the final drying. Equation 2:

$$M_{id} = M_i \times (100-(\%humidity/100)) \quad \text{Equation 2}$$

$$DI = \left[\frac{M_{id}-M_{fd}}{M_{id}}\right] X100 \quad \text{Equation 3}$$

The evaluation was performed for the times of: 0, 10, 30, 60, 90, 120, 180, 240 and 300 minutes, with n = 10.

Degree of crosslinking with genipin - absorption Spectro photometry: The degree of crosslinking with genipin between chitosan and type I collagen was determined by the ninhydrin assay and was defined as the ratio between the amino groups consumed in the chitosan (Ch), collagen (Col), chitosan-collagen (Ch / Col) and chitosan-collagen genipine (Ch / Col / Gen) with increasing concentrations of genipin 0.1 %, 0.25 %,

0.50 %, 0.75 % and 1.00 % v/v by crosslinking and free amino groups in the biomatrices. The amino acid L-arginine was used as standard in different concentrations (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mg mL^{-1}) for the construction of the analytical equation. Ten measurements were performed for each biomatrix group (n = 10). The amount of free amine groups in the biomatrices was determined by the optical absorbance of the solution at 570 nm, with "white" subtraction in a Microplate Spectrophotometer apparatus (BioTek® Instruments, USA).

Statistical analysis

Statistical analysis of the data was performed and interpreted by the author of the present study. For data analysis a database was built in the Microsoft Excel spreadsheet which was exported to the Minitab 17 statistical program. A common descriptive statistical analysis and Anderson-Darling normality test were performed for all variables and controls, with reference p> 0.10 as "normal". As there were continuous and categorical predictors (chitosan / collagen biomaterials crosslinked with increasing genipin concentrations of 0.10 %, 0.25 %, 0.50 %, 0.75 % and 1.00 % v/v) and the response predictors (chitosan, collagen and chitosan / collagen biomatrizes), linear regression and residual Durbin-Watson analysis were applied. For all linear regression tests, alpha level lower than 0.05 was adopted as significant. For Durbin-Watson residue analysis, the reference significance level was 0.05, adopting as acceptable range of independence 0.95 <dw <1.54 (according to the Durbin-Watson standard table, dU <dw <4-dU), with two explanatory variables for sample size of n = 10.

RESULTS

Study of Normality (Anderson-Darling)

In all analyzes of the present study, according to figures 1, 5 and 8, it was observed that all the samples presented normal distribution, with p> 0.10, followed by parametric statistical analysis.

Degree of Swelling

Figure 2 shows that the chitosan bio matrix, due to its higher number of hydrophilic groups, showed a higher degree of swelling, followed by collagen, chitosan-collagen and collagen chitosan-collagen bio matrices with a gradient of 0.10 % to 1.00 %. Due to the increase in the rigidity of these biomaterials with the increase of genipin concentration, the 0.75 % bio matrix presented moderate stiffness and swelling degree around 80.0 %, pointing to be chosen for future studies. Statistical regression analysis between continuous and categorical predictors (chitosan / collagen biomaterials crosslinked with increasing genipine concentrations of 0.10 %, 0.25 %, 0.50 %, 0.75 % and 1.00 % v/v) and the predictors re sponse (chitosan, collagen and chitosan / collagen biomatizes) showed that there was no statistical significance between the continuous and categorical predictors in relation to the biomarkers response chitosan and collagen, evidencing that there was no difference in the degree of swelling, with p> 0.05, however, already in relation to the predictor bio matrix response of chitosan/collagen was statistically significant, with p <0.05, as shown in figure 3. In the Durbin-Watson residual analysis, all correlation results between the degree of swelling of the bio matrices were within the acceptable range of independence 0.95 <dw <1.54 (according to the Durbin-Watson standard table, dU <dw <4-dU), with two explanatory variables and a sample size of n = 10. Therefore, there was no relationship of dependence (significance) between the data analyzed. Thus, the results are confirmed by figure 4, where the residues appear to follow a straight line. There is no evidence of discrepant points or unidentified variables; the residues appear to be randomly scattered around zero. There is no evidence of non-constant variance, absent terms, discrepant points or influential points; the histogram does not follow a normal curve; the residues appear to be randomly scattered around zero. There is no evidence that the error terms are correlated with each other.

Biomatrix Degradation Index

Figure 6 shows the rate of weight loss of the matrices during immersion in a solution of human blood. Matrices with increasing gradient of genipin content of 0.10 % to 1.00 % present in this sequence, a tendency of reduction in the degradation rate since the biomatrix that has lower levels of crosslinker tends to increase the degradation rate with time.
Statistical regression analysis between continuous and categorical predictors (chitosan / collagen biomatrices crosslinked with increasing concentrations of genipin 0.10 %, 0.25 %, 0.50 %, 0.75 % and 1.00 % v/v) and the response predictors (chitosan, collagen and chitosan / collagen biomatrices) showed that there was no statistical significance between the continuous and categorical predictors in relation to the biomarkers response chitosan and collagen up to the concentration of 0.25 % v/v genipin, showing that there was no difference in the degree of degradation, with p <0.05. However, since 0.50 % v/v of genipin, there was a statistical difference, with p> 0.05, as shown in figure 7. In the Durbin-Watson residual analysis, all correlation results between the degree of swelling of the biomatrices were within the acceptable range of independence 0.95 <dw <1.54 (according to the Durbin-Watson standard table, dU <dw <4-dU), with two explanatory variables and a sample size of n = 10. Therefore, there was no relationship of dependence (significance) between the data analyzed. In this way, the waste appears to follow a straight line. There is no evidence of discrepant points or unidentified variables; the residues appear to be randomly scattered around zero. There is no evidence of non-constant variance, absent terms, discrepant points or influential points; the histogram does not follow a normal curve; the residues appear to be randomly scattered around zero. There is no evidence that the error terms are correlated with each other (Figure 4).

Degree of cross-linking of bio matrix with genipin gradient

Before measurements of the degree of cross linking with genipin, the standard curve (analytical equation) was made with the amino acid L-arginine with a concentration gradient of 1.0 to 8.0 mg mL^{-1}. Figure 9 shows the degree of crosslinking in chitosan, collagen, chitosan/collagen and chitosan/collagen crosslinked with increasing concentrations of genipin of 0.10 %, 0.25 %, 0.50 %, 0.75 % and 1.00 %. The degree of cross linking ranged from 80.0 % to 6.3 %, according to the number of free amino groups and the concentration of genipin, as shown in Table 1. Statistical regression analysis between continuous and categorical predictors (chitosan/ collagen biomaterials cross linked with increasing genipin concentrations of 0.10 %, 0.25 %, 0.50 %,

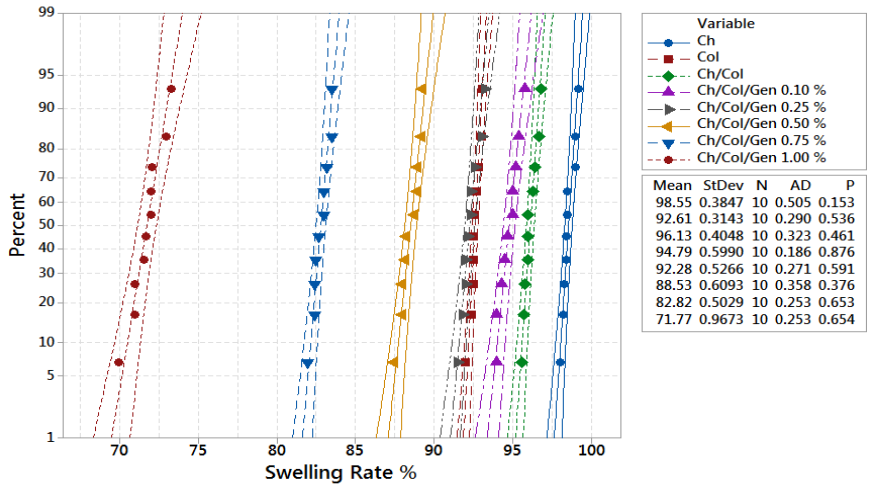

Figure 1. Normality test for the swelling test, with p> 0.10 as significant

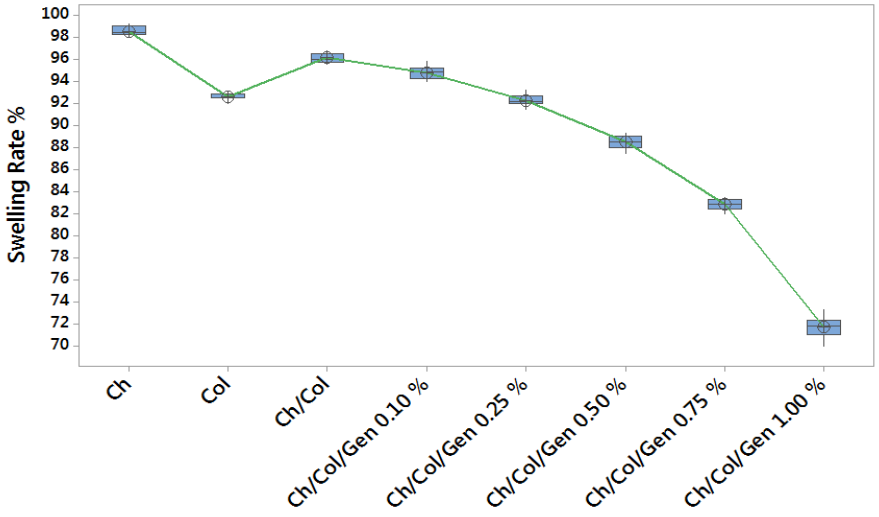

Figure 2. Box-Plot model showing statistical values of mean, standard deviation and decline of the swelling curve with increasing genipin concentration

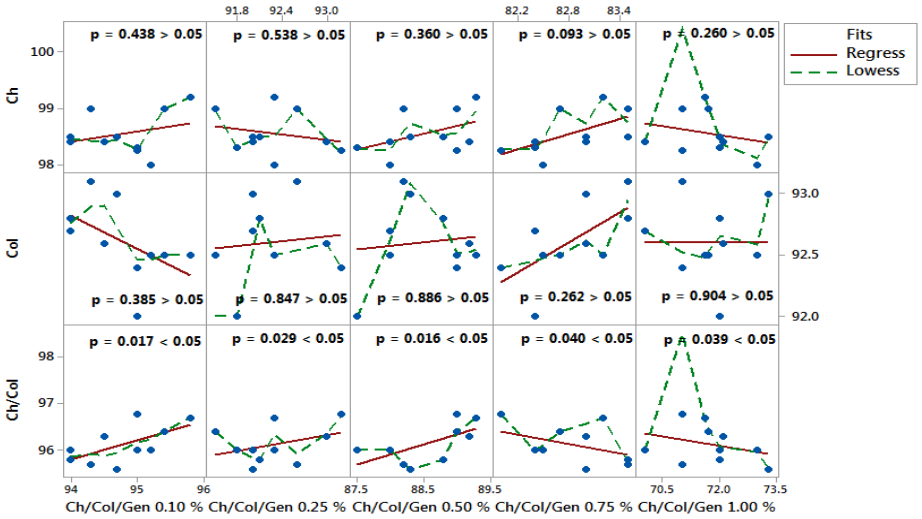

Figure 4. Graph Matrix-Plot model showing the results of the regression analysis between the continuous / categorical predictors and response in the swelling test

0.75 % and 1.00 % v/v) and the predictors response (chitosan, collagen and chitosan/collagen bio matrices) showed that there was no statistical significance between the continuous and categorical predictors in relation to the biomarkers response chitosan and collagen, evidencing that there was no difference in the degree of swelling, with p> 0.05. However, in relation to the biomaterial response of chitosan / collagen, there was a statistical significance, with p <0.05, as shown in Figure 10. In the Durbin-Watson residual analysis, all correlation results between the degree of swelling of the bio matrices were within

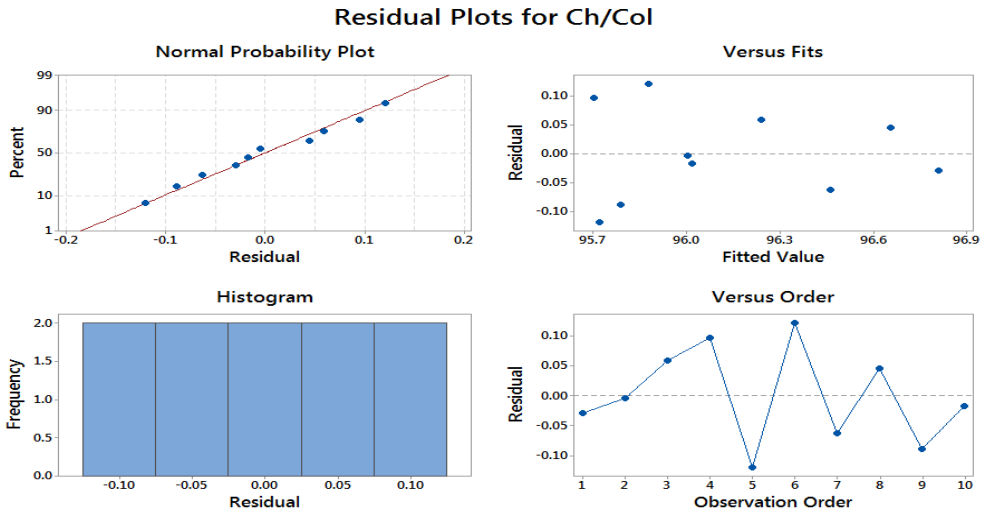

Figure 04. Graphs representative of the three trials of the present study, showing in a generic way the results of the residual analysis

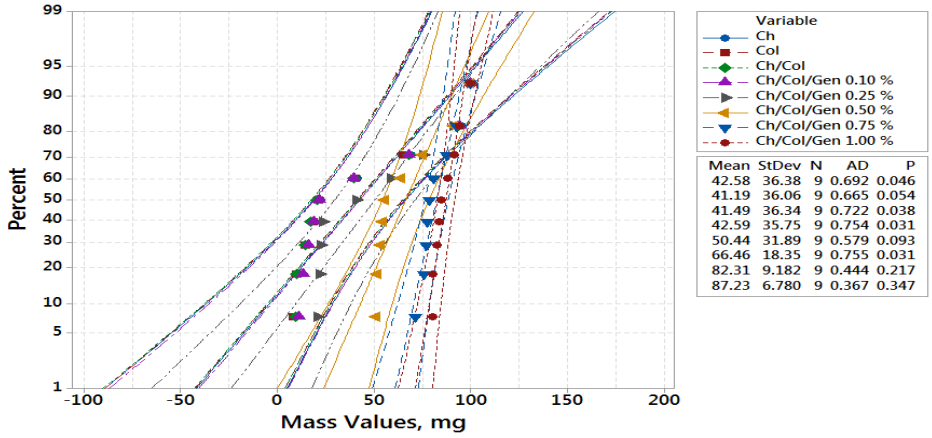

Figure 05. Normality test for the degradation index test, with p> 0.10 as significant

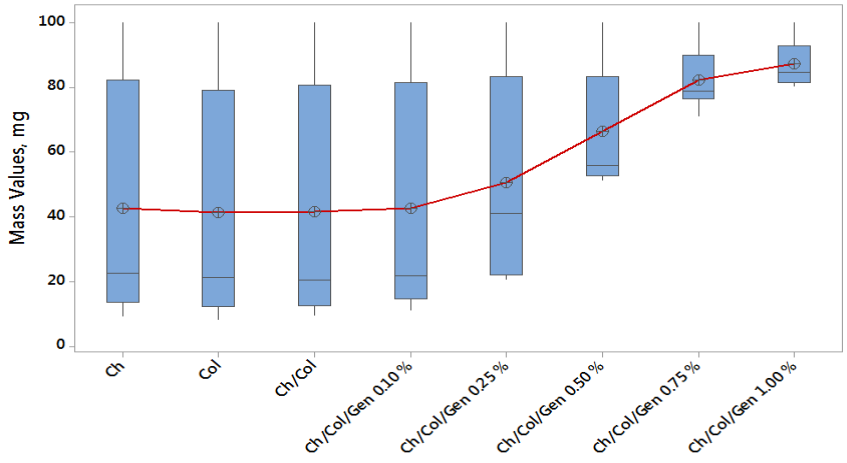

Figure 06. Box-Plot model showing statistical values of mean, standard deviation and decrease of the degradation of the biomatrices with the increase of the concentration of genipin

the acceptable range of independence 0.95 <dw <1.54 (according to the Durbin-Watson standard table, dU <dw <4-dU), with two explanatory variables and a sample size of n = 10. Therefore, there was no relationship of dependence (significance) between the data analyzed. Thus, the results are confirmed by Figure 4, where the residues appear to follow a straight line. There is no evidence of discrepant points or unidentified variables; the residues appear to be randomly scattered around zero.

There is no evidence of non-constant variance, absent terms, discrepant points or influential points; the histogram does not follow a normal curve; the residues appear to be randomly scattered around zero. There is no evidence that the error terms are correlated with each other (figure 4). These results demonstrated that genipin is favorable cross linking reagent for collagen and chitosan, as it can efficiently crosslink amino groups, even at low concentration.

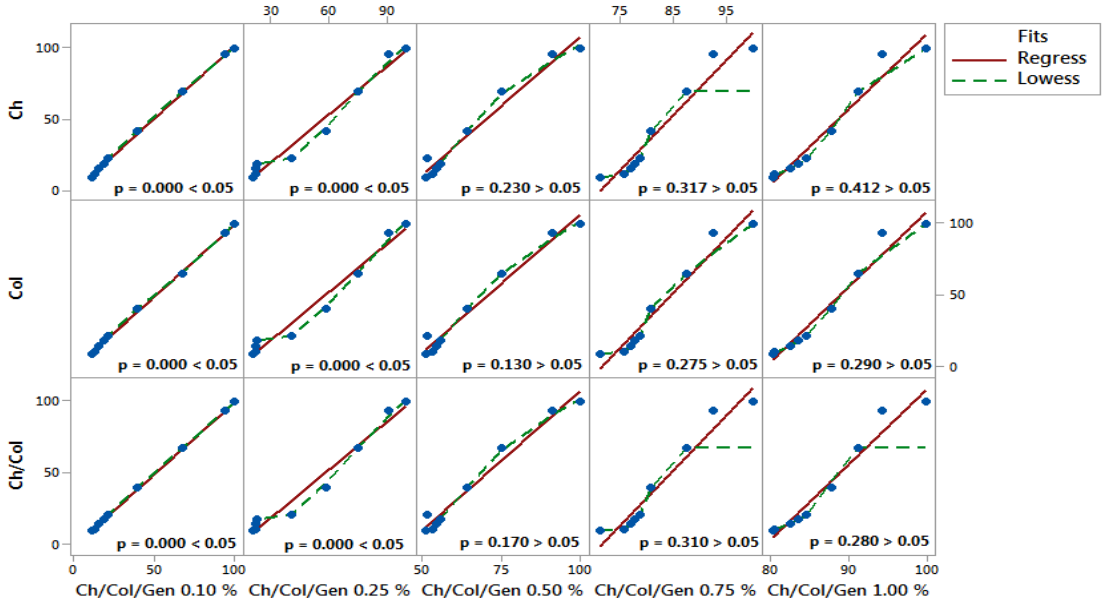

Figure 7. Graph Matrix-Plot model showing the results of the regression analysis between the continuous / categorical predictors and the response in the degree of degradation assay

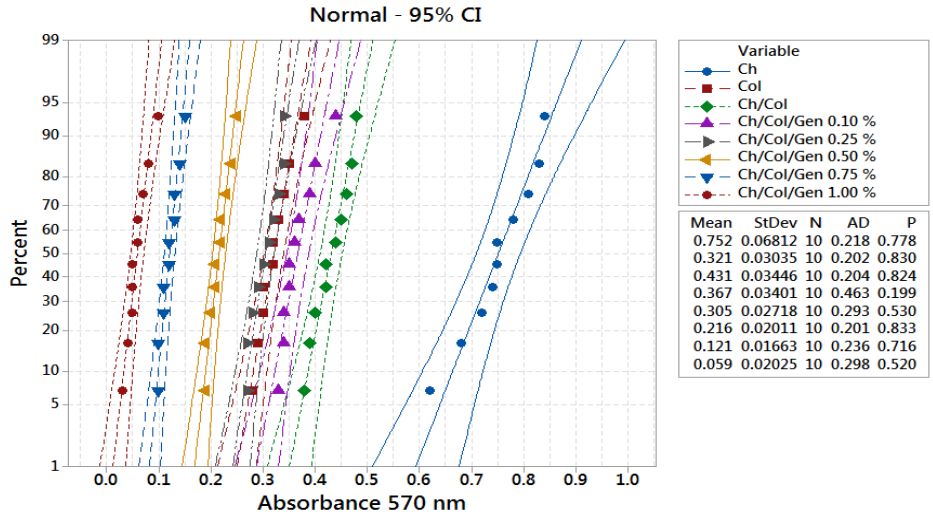

Figure 8. Normality test for the cross linking grade assay, as p> 0.10 as significant

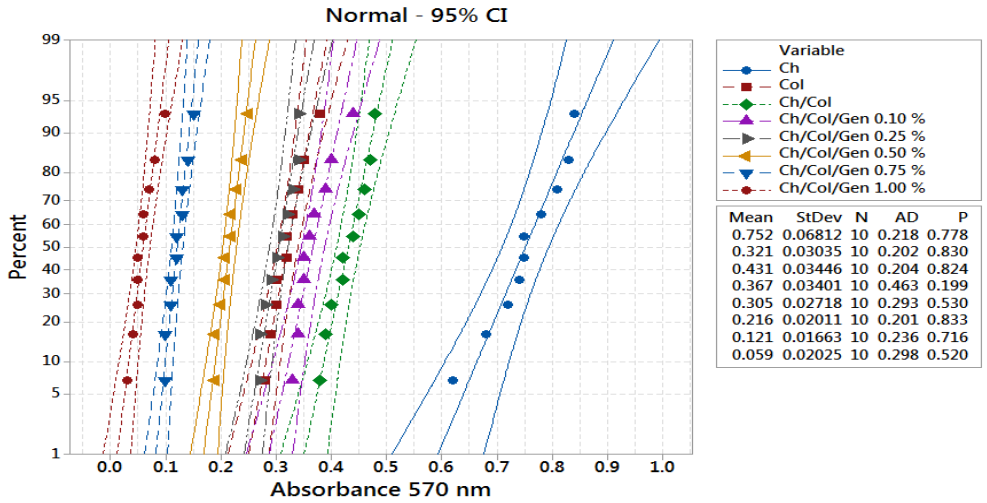

Figure 9. Box-Plot model showing statistical values of mean, standard deviation and decline of the degree of cross-linking with increasing genipin concentration

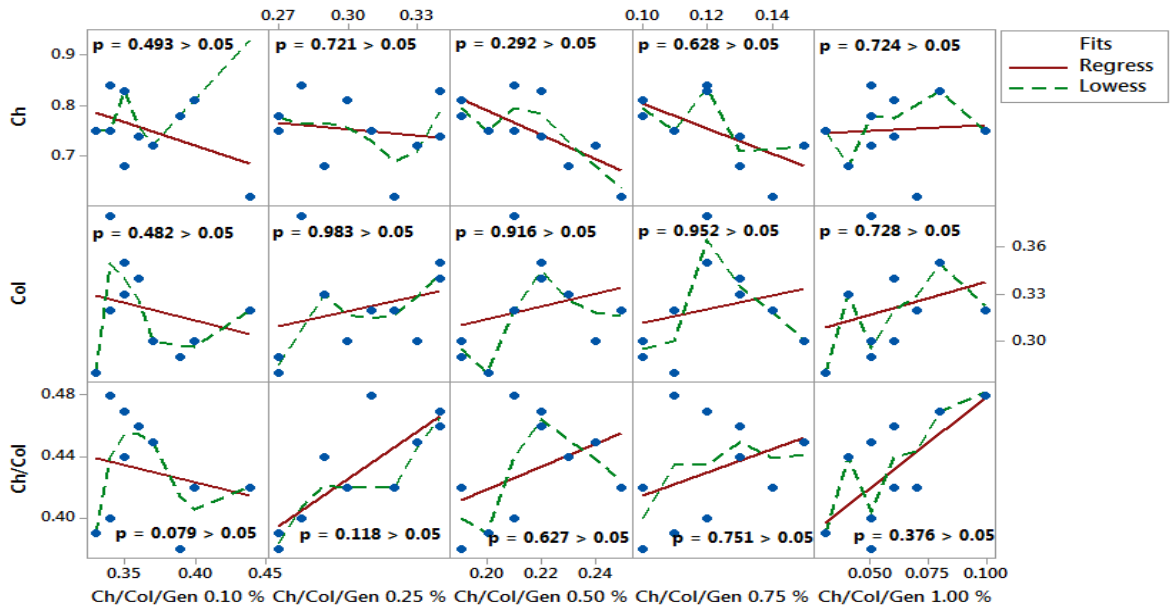

Figure 10. Graph Matrix-Plot model showing the results of the regression analysis between the continuous / categorical predictors and response in the crosslinking degree test

Table 1. Values as a percentage of the decrease in the degree of crosslinking with increasing genipin concentration

Biomatrices	Degree of crosslinking%
L-arginina (standard)	100,0
Chitosan	80,0
Collagen	34,1
Chitosan/Collagen	45,9
Chitosan/Collagen/Genipin (0.10 % v/v)	39,0
Chitosan/Collagen/Genipin (0.25 % v/v)	32,4
Chitosan/Collagen/Genipin (0.50 % v/v)	23,0
Biomatrices	Degree of crosslinking%
L-arginina (standard)	100,0
Chitosan	80,0
Collagen	34,1
Chitosan/Collagen	45,9
Chitosan/Collagen/Genipin (0.10 % v/v)	39,0
Chitosan/Collagen/Genipin (0.25 % v/v)	32,4
Chitosan/Collagen/Genipin (0.50 % v/v)	23,0
Chitoan/Collagen/Genipin (0.75 % v/v	12,9
Chitosan/Collagen/Genipin (1.00 % v/v)	6,3

In addition, as the concentration of genipin increased, more amino groups were cross-linked. The region highlighted in blue in Table 1 shows t he bio matrix with 0.75 % v/v of genipin that was the one that presented the best physicochemical characteristics in the present study and was therefore the bio matrix selected for the biological studies of other works.

DISCUSSION

Biomatrix in tissue engineering

Tissue engineering is a tool that enables the creation of a suitable biological niche for the construction and regeneration of any tissues and organs (Muzzarelli *et al.,* 2016; Varoni *et al.,* 2017; Bonfield, 2006; Breyner *et al.,* 2010). For this, autogenous or allogeneic graft pathways are used, with and without the use of cells (Irioda *et al.,* 2016). For example, biomaterials such as chitosan, collagen and polyhydroxybutyrate, which are biodegradable, biocompatible and non-toxic, can act in the controlled release of drugs, gene transfection and tissue regeneration (Friedenstein, 1976). According to the Conference of the National Institute for the Development of Health Consensus in 1982, biomaterials are beneficial organic compounds, or a combination thereof, that can be used for a period of time, wholly or partially as part of a system that treats, replace any tissue, organ or function of the human body (Planat Bernard *et al.,* 2004). The great challenge is to understand that the science of biomaterials is multidisciplinary and its application requires adequate processing, sterilization and structural modifications that favor interaction with the tissue of interest. There are several biomaterial manufacturing models. These models follow geometric representations that must be in agreement with the type of tissue or organ of interest. To satisfy the diversity of tissues and organs, biomaterials can be manufactured in the form of porous bio matrices, thin superimposed layers, beads and in the form of elongated yarns (Breyner *et al.,* 2010). The biomaterial type also directs the type of cell line it can store and stimulate proliferation or differentiation from stem cells (Zazakowny *et al.,* 2016; Zotarelli Filho *et al.,* 2015, Chan *et al.,* 2009), as presented with the use of chitosan-collagen genipin in the present study. Thus, bioengineering and cell therapy work together for regenerative medicine, favoring and improving biological conditions to accelerate repair and tissue regeneration, and thus maintain tissue homeostasis naturally.

This condition is maintained because the required cellular elements, cell proliferation and differentiation factors, and supramolecular structures are provided which guarantee the functional stereochemical organization of the generated tissues and their systemic integration (Chen *et al.,* 2006).

Physical chemical Studies of Bio matrices

Degradation of chitosan-collagen-genipin matrices in the presence of human blood in culture media decreased as the degree of cross-linking of the bio matrix with genipin increased. This is because of the greater energy required for depolymerization of the polymer chains crosslink (crosslink covalent) than that showed low level of cross linking agent. The results below show the preferred cross linking via NH2 (chitosan) instead of hydroxyl (collagen) therefore is expected in a mixture degradation kinetics for the blends, the collagen that is physically crosslinked undergoes rapid solvation and chitosan/collagen are covalently crosslinked via deploy merization slower degradation. In comparison with the studies by Chiono *et al.* (2008), matrix solubility increased as the degree of cross linking with genipin was also increased, due to the increased vulnerability to dissolve the matrices by increasing the degree of dissociation of chitosan and collagen with protonation of the groups aminos. This revealed that the best concentration of genipin for the manufacture of the bio matrices was 0.75 % (v/v), similar to the results reported by Baldwin; Kiick (2010). Thus, when the degree of crosslinking with genipine was less than 0.50 % v/v biomaterial was very fragile, with low resistance, and when the genipin concentration was greater than 1.00 % v/v, the biomaterial ruptured and pieces of " flakes "left the bio matrices. The morphology of the matrix structure as well as its porosity were determined by scanning electron microscopy, showing the presence of spaces (pores) for the passage of nutrients, gases and cellular metabolites, as well as cell adhesion and proliferation (Ko *et al.,* 2010; Langer and Vacanti, 1999).

The biocompatibility of the matrices was due to the adhesion, proliferation and modulation of the cellular activities of CTMA and macrophages by the activity of alkaline phosphatase and nitrogen oxide, respectively, as a similar work done by Planat Bernard *et al.* (2004). The miscibility of chitosan with collagen was due to electrostatic interactions in ion-ion, dipole-dipole and dipole-dipole interactions, Van der Waals interactions, π-electrons and charge transfer complexes, forming ionic bonds of hydrogen and covalent bonds between the polymeric components, as well as free Gibbs free energy ($\Delta G < 0$), according to the Domard literature; Taravel (1995) and Israelachvili (2011)(Israelachvili, 1973, Israelachvili, 2011), in the mixture among other polymers, although these are of high molecular weight. The hydroxyproline (OH-) groups of collagen can form hydrogen bonds between the chains and interactions with other side groups are important in the formation of collagen fibers. These side groups may also form hydrogen bonds with -OH and NH_2 of chitosan. In addition, the terminal groups -COOH and -NH2 may form hydrogen bonds with -OH and $–NH_2$ of the chitosan. This mixture of polyactions (chitosan) and polyanions (collagen) may have led to spontaneous aggregation and release of counterions, causing an entropy gain ($\Delta S > 0$) (Israelachvili, 2011). The presence of the counterions, as well as the presence of the cations and anions of the individual polymer chains can stimulate the cellular activities of human tissues and thus favor tissue regeneration (Zhan *et al.,* 2012).

Action of the intermolecular forces in the constitution of the biomatrix

Chitosan reacts easily by nucleophilic attack of its amino groups with carbonyl compounds, forming a covalent bond with some collagen and genipin clusters (Antonov and Moldenaers, 2012; Arof *et al.,* 2010). The miscibility of chitosan is due to electrostatic interactions in the ion-ion, dipole-ion, and dipole-dipole interactions, Van der Waals interactions, π-electrons and charge transfer complexes, forming ionic, hydrogen and covalent bonds between the polymer components, producing a negative Gibbs ($\Delta G < 0$) free energy in the mixture, despite the high molecular weight of the polymers (Alsarra, 2009; Domard and Taravel, 1195; Drago, 1977; Israelachvili, 2011). The hydroxyproline (-OH) groups of collagen make hydrogen bonds between the chains and the interactions with the other side groups of the collagen are important in the formation of fibers. These side groups also form hydrogen bonds with -OH and NH_2 of chitosan. In addition, the collagen-COOH and -NH2 end groups of the collagen may also form hydrogen bonds with -OH and $–NH_2$ of chitosan (Zhan *et al.,* 2012; Hill, 1963). The two polymer chains of chitosan-collagen can intertwine, forming a complex with higher viscosity (Hill, 1963). Mixtures of polyacites (chitosan) and polyanions (collagen) lead to spontaneous aggregation and the release of counterions, leading to an entropy gain ($\Delta S > 0$) (Israelachvili, 1973).

Conclusion

As the degree of interlocking with genipin increased, the biomatrices became more rigid, reducing the degree of swelling and free amino groups. Thus, through the present investigation, the chitosan/collagen biomatrix with 0.75 % v/v genipin provided the best physico-chemical properties for future cell and clinical studies.

Declaration of Potential Conflict of Interest

The authors declare no conflict of interest.

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