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Original Research

Assessment of the Processing Method for the Development of Hybrid Biopolymer-Based Scaffolds

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Abstract

The present study deals with the development of scaffolds based on gelatin (G) and/or chitosan (CH) through modifications in the central processing method. This process consists of the fabrication of a hydrogel which is, then submitted to a freeze-drying stage. To compare the effects of the different modifications, the mechanical properties of the various systems were characterized, employing both dynamic compressive strain and frequency sweep tests. In addition, their porosity as well as the structure and fiber distribution, using an analytic model and scanning electron microscopy (SEM), were also evaluated. The obtained results demonstrated a strong dependence on the properties by the scaffolds with both the modifications introduced in the processing method as well as the proportion of materials used (G and CH). Furthermore, the properties were found to improve for systems with a high chitosan content after being submitted to a heat treatment at 50 °C with agitation.

Keywords
Gelatin; chitosan; freeze-drying; scaffold; biopolymers

1. Introduction

The concept of Tissue Engineering (TE) was introduced in the eighties, based on the pioneer studies carried out by Yannas and, especially, Vacanti, who conceived the idea of cell support to grow and form a tissue [1]. However, TE was not defined until the year 2000, when researchers working in the field agreed to establish TE as the science of fabrication and design of new tissues intended for the functional restoration of altered organs as well as a substitution of structures damaged by trauma or diseases [2]. TE is thus a multidisciplinary field in which different branches of science like Biology, Chemistry, Medicine and even Engineering interact. Three main elements composing TE are cells, growth factors, and scaffolds, the latter being the platform which provides anchorage and adhesion for cells to proliferate and differentiate. Among scaffolds, those with a certain porosity, have been widely employed in TE studies concerning tissue regeneration to provide a three-dimensional structure. Regardless of the application, the materials used and the structure inside the scaffold have a significant effect on cell activity. For this reason, the selection of a proper scaffold is essential for optimum cell growth [3]. In addition, scaffolds have to satisfy certain conditions such as biocompatibility, having a macroscopic structure similar to the tissue to be replaced, some degree of mechanical resistance and an internal porous structure with specific pore size and interconnectivity. These characteristics affect cell activity as proper pore size and distribution guarantee good insertion and cell growth through the scaffold, improving its bio integration [4].

Scaffolds are made of materials that match the structural function of the organ or tissue to be replaced. Among the various polymers, synthetic ones are the most widely used [5]. However, the most attractive options are based on proteins and polysaccharides due to their high biocompatibility, and thus, scaffolds made of collagen, fibrin, elastin, alginate, chitosan, etc. can also be found. Moreover, among all available materials, collagen and chitosan stand out for their ability to adopt many different structures, similar to the biological ones.

Collagen is the main structural protein in the extracellular space of the connective tissue in the human body. As the main component, it is the most abundant protein in mammals, being between 25% and 35% of the whole protein content in the body. Collagen is composed of amino acids that form triple helices of elongated fibers [6]. This compound is mainly present in fibrous tissues such as tendons, ligaments, and skin [7]. Apart from native collagen protein, denaturalized collagen, known as gelatin, has many applications, especially in the pharmaceutic and food industry [8].

Chitosan is obtained from chitin through a deacetylation process; chitin is very abundant in nature. It is a part of the cellular walls in fungus, the arthropod exoskeleton and some organs in many other animals, like *Annelid quetas* [9]. Thus, it has a vast range of applications as a flocculant agent in water treatment, wound disinfectant in medicine, and as a thickener in the food industry. Furthermore, chitosan is also useful in medicine, not only because of its antimicrobial properties but for being a biocompatible and biodegradable substance [10]. Therefore, it is often used for manufacturing biodegradable suture threads that vanish once the wound is closed. In addition, it is

a very powerful procoagulant and added to gauzes and bandages, in the United States and Europe, to avoid hemorrhages.

Gelatin and chitosan-based combined scaffolds can suit different applications in tissue engineering by selecting the proper processing method. Collagen/gelatin and porous chitosan scaffolds developed by the mechanical spinning of chitosan and mixing collagen and gelatin solutions followed by freeze-drying and crosslinking of polymers have demonstrated high porosity, good swelling capacity and suitable mechanical, antimicrobial and antioxidative properties, which are the desirable properties for wound healing applications [11]. Moreover, beta-glycerophosphate was used to initiate gelation of chitosan-collagen composite hydrogels which were able to encapsulate adult human bone marrow-derived stem cells exhibiting high viability [12]. The presence of collagen is associated with increased cell spreading and proliferation and chitosan with improved osteogenic differentiation. Thus, such materials can be used for cell encapsulation and delivery, or as in situ gel-forming materials for tissue repair.

Despite a large number of techniques that can be used to manufacture scaffolds, they are traditionally obtained by phase separation [13]. This technique involves preparing a solution by inducing its gelation, followed by the sublimation of the solvent by lyophilization. In addition, modifying the processing conditions by including additional processes such as heat treatment, or adding different solvents or compounds that favor crosslinking or reticulation of the structure (for example, glutaraldehyde or elastin), leads to a vast variety of structures with many microstructures [14, 15].

The primary purpose of this work is to study the influence of different processing factors in the properties of biopolymer-based sponge-like scaffolds. Also, the possible synergy between proteins and polysaccharides has been evaluated by producing scaffolds with different gelatin-chitosan mixtures. Thus, the main objective of the present study was the development of gelatin (G) and/or chitosan (CH)-based scaffolds using a manufacturing technique derived from the phase separation process. In this way, scaffolds with 1 wt% polymer content were obtained by different variations in the processing technique and different G/CH proportions. The mechanical properties (by dynamic strain and frequency sweep tests) and microstructure (by porosity measurements and scanning electron microscopy) of the samples were measured to evaluate the effect of the composition and fabrication process in the scaffolds.

2. Materials and Methods

2.1 Materials

The scaffolds were prepared using gelatin and/or chitosan. Gelatin (G) protein was obtained from fish skin (type B, Bloom 80–120 g) provided by Henan Boom Gelatin Co. Ltd (China) with a protein content above 95 wt.%. Chitosan (CH) is a low molecular weight polysaccharide (MW = 130.000 $g \cdot mol^{-1}$), procured from Sigma Aldrich (Germany).

Other reactants such as acetic acid and glycerol, used as a plasticizer, were provided by Panreac Química, S.A. (Spain).

2.2 Scaffolds Production

The scaffolds were manufactured following a method described by Angulo et al. [16]. The solutions with 1 wt.% biopolymer (50G–50CH proportion) containing 0.05M acetic acid as a solvent were prepared. This proportion was selected based on previous studies showing a better combination of mechanical and structural properties [17]. A biopolymer solution was favored using a heat treatment under magnetic agitation for 2 h at 50 °C. Later, 0.3 wt.% glycerol was added, and the pH was adjusted to 5 using 4M NaOH. The different systems obtained were transferred to a glass Petri dish and frozen at –40 °C for 2 h before the lyophilization step at –80 °C and 0.01 bar for 24 h (LyoQuest, Telstar, Spain).

This system (50G–50CH proportion) was subjected to certain variations during the process to evaluate its effect. Three modifications were introduced: avoiding glycerol addition, using a lower temperature during agitation (from 50 to 20 °C) and avoiding agitation (Table 1). In this later situation, to evaluate the effects of agitation alone, an oven was used to increase the temperature to 50 °C. Finally, the processing technique which resulted in better mechanical properties was selected to study the effects of composition (G-CH proportion) on the properties of the scaffolds.

Table 1 Different systems produced with the different processing conditions.

CODE	**Glycerol**	**Temperature (°C)**	**Stirring**
A (Reference)	YES	50	YES
B	NO	50	YES
C	YES	20	YES
D	YES	50	NO

2.3 Scaffolds Characterization

2.3.1 Mechanical Properties

Mechanical characterization was carried out using a dynamic-mechanical rheometer RSA3 (TA Instruments, USA) in compression mode with a circular plate-plate sensor (diameter: 15 mm). Two different tests were performed: a strain sweep test between 0.002% and 2% implemented at a constant frequency of 1 Hz to determine the linear viscoelastic range (LVR) and the critical strain (γ_c) through the stress-strain curve. Subsequently, a frequency sweep test between 0.02 and 20 Hz with a strain within the LVR (below γ_c) was carried out to obtain information regarding the elastic (E') and viscous (E'') moduli along with their relationship (loss tangent, tan (δ)=E''/E') in the whole studied frequency range. Besides, to accomplish a more efficient comparison between the different systems, E' and tan (δ) at 1 Hz (E'_1, $\tan(\delta)_1$, respectively) were selected.

2.3.2 Porosity

The scaffolds porosity (ε) was obtained following the methodology described by Al-Munajjed et al. [18]. Individual porosity was calculated using equation 1:

$$\varepsilon\ (\%) = (1 - \frac{\rho_s}{\rho_m}) \cdot 100 \quad (1)$$

where ρ_s is the density of the scaffold (calculated from the weight and volume of each scaffold), and ρ_m is the density of the G-CH mixed system [19], considering the density of gelatin (0.68 g·cm^{-3}) and chitosan (1.456 g·cm^{-3}), assessed by Pentapyc 5200e (Quantachrome Instruments, USA).

2.3.3 Morphological Properties

The microstructure and fiber distribution were determined using scanning electron microscopy (SEM) (Zeiss AURIGA, USA) including a secondary electron detector at an acceleration voltage of 20 kV. Since the scaffolds are insulators, they were coated with a thin layer of gold and palladium by sputtering. Following this, the scaffolds showed conductive properties [20]. In addition, to ensure the scaffold fixation and prevent loss in its structure, a treatment employing 1% osmium tetroxide vapor for 8 h was carried out.

2.4 Statistical Analysis

Statistical analysis was carried out for all of the selected parameters. The one-way analysis of variance (ANOVA) was implemented using three different independent measures. Statistical parameters, such as mean and standard deviation, were also calculated. Furthermore, a mean comparison test was carried out to evaluate significant differences ($p < 0.05$).

3. Results and Discussion

3.1 Influence of the Processing Technique

The effect of the processing technique on the final properties of the different scaffolds was studied. For this purpose, the composition used in the system was the 50G–50CH proportion, following the processing technique, explained in Section 2.2., following the standard stages (system A). The variations introduced in the process were avoiding the glycerol addition (system B), lowering the temperature during agitation (from 50 to 20 °C) (system C) and avoiding agitation (system D).

3.1.1 Mechanical Properties

Figure 1 shows the mechanical tests of the systems obtained from different processing conditions. Figure 1A shows the evolution of the elastic modulus (E') in the whole frequency range. It depends on the processing conditions that whether E' becomes more dependent on the frequency used or not. A stronger dependency was observed for lower temperatures (C) and systems with no agitation stage (D). Although these systems are the most dependent on the elastic modulus, they also provide higher E' values. Conversely, no significant differences could be observed with no glycerol addition (B) compared to the reference system (A), being mainly stable in the whole frequency range studied. These variations in the mechanical behavior of the different scaffolds could be due to the different protein structuration. When lower temperatures are used during agitation (C) or this stage is avoided (D), it seems that it is more difficult for the system to rearrange its structure, possibly due to the lower solubility of the used materials (G and CH), resulting in a less stable system. However, whether glycerol is added or not (A and B, respectively),

it does not affect the system, and thus glycerol is not required to obtain a stable system. This explains why most investigations regarding this kind of scaffolds do not use glycerol in their composition [16, 21].

Figure 1B shows the elastic moduli (E'_1), the loss tangent (tan (δ_1)) at 1 Hz, and the critical strain (γ_c) of the differently processed systems. It was observed that the systems with lowered temperature (C) or avoided agitation stage (D), showed higher E'_1 results, as noted previously. The reason for this behavior could be that these stages favor the scaffold structuration resulting in stiffer materials, making them more susceptible to the frequency used. However, the loss tangent at 1 Hz (tan (δ_1)) is below 1 in all cases indicating that all systems present a strong solid character, an essential characteristic that allows cells to grow inside them. This behavior can also be observed in systems similar to an earlier study [22]. Regarding the critical strain (γ_c), a higher value was obtained when the system was kept under agitation for 2 h at 50 °C (A).

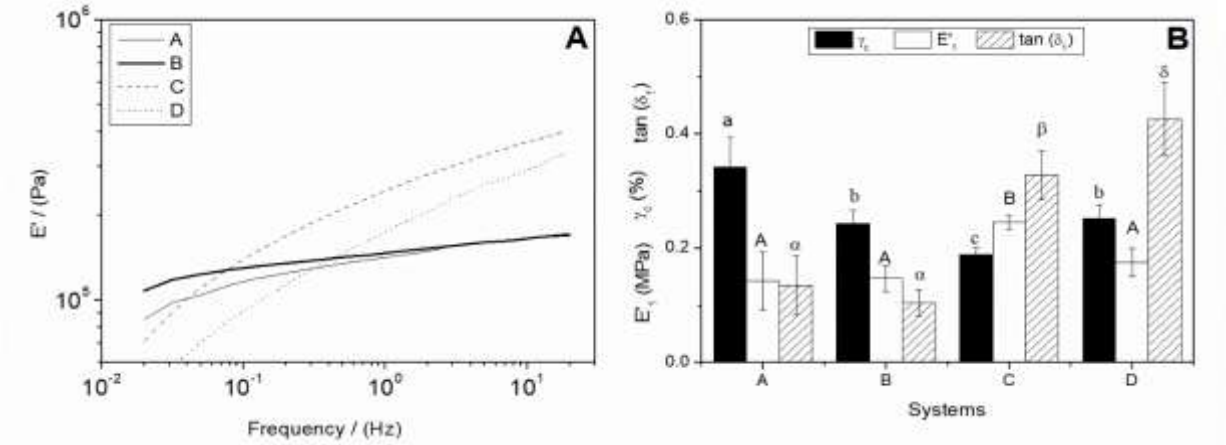

Figure 1 (A) Frequency scanning and (B) critical strain (γ_c), loss tangent and elastic module at 1 Hz ($\tan(\delta)_1$ y E'_1) of the scaffolds manufactured with different processing conditions and a 1:1 gelatin/chitosan proportion (50G–50CH). Differently labeled columns are significantly different (p <0.05).

3.1.2 Porosity

Table 2 shows the porosity values for the different obtained systems. It can be observed that porosity is above 96% in all systems, i.e., an acceptable percentage for cell proliferation [16]. In the cases where the reference method was modified, slightly higher porosities were obtained, with no significant differences from the three modifications. This improved porosity explains a higher elastic module obtained by the methods C and D as they present more free volume. However, they showed poor stability against frequency due to their brittle structure. Finally, the reference system (A) showed higher stability among its mechanical properties (E') and a higher critical strain (γ_c). Thus, the reference processing method was chosen to study the effect of the scaffold composition.

Table 2 Porosity values for the systems produced with different processing conditions, using the 50G/50CH system as a reference; and porosity values obtained with different gelatin/chitosan ratio. The columns with different superscript letters correspond to the values significantly different ($p<0.05$).

SYSTEMS		**ε (%)**
Variables	A	95.9 ± 0.8^{a}
	B	98.5 ± 0.2^{b}
	C	97.7 ± 0.1^{c}
	D	98.8 ± 0.2^{b}
G-CH ratio	0G-100CH	97.8 ± 0.3^{c}
	25G-75CH	97.1 ± 0.5^{d}
	50G-50CH	95.9 ± 0.7^{a}
	100G-0CH	95.9 ± 0.7^{a}

3.2 Influence of the Gelatin-Chitosan Ratio

Scaffolds with different gelatin-chitosan ratios were obtained using the A reference processing method (heat treatment at 50 °C for 2 h with agitation) to study the effects of each biopolymer on the final properties of the obtained systems.

3.2.1 Mechanical Properties

Figure 2 shows the mechanical tests of the scaffolds obtained from different G and CH proportions. Figure 2A shows the evolution of the elastic modulus (E') with the frequency to study the influence of the G-CH proportion on the mechanical properties of the scaffolds. The higher the CH concentration, the systems exhibited higher E'. Furthermore, although the properties of all systems depend on the frequency used, it is stronger for systems with higher CH content. This behavior is in agreement with Figure 1, that is, the systems with higher CH content will probably present higher porosity, containing more free volume resulting in improved elasticity but poor stability. Thus, they are more susceptible to frequency variations. Similar behaviors can be observed in related work [21-23].

On the other hand, Figure 2B shows the elastic moduli and loss tangent values at 1 Hz (E'_1 and tan (δ_1), respectively), as well as the critical strains (γ_c) of the different systems. E'_1 values were observed to increase for systems with a higher chitosan concentration, as could be noted previously. However, tan (δ_1) was below 1 for all systems, being mainly elastic. Finally, higher γ_c was observed with the 0G–100CH system, indicating that an increase in the chitosan content is associated with an increase in the mechanical properties. Although it is not the most stable system with the frequency, it exhibits higher elastic modulus and critical strain.

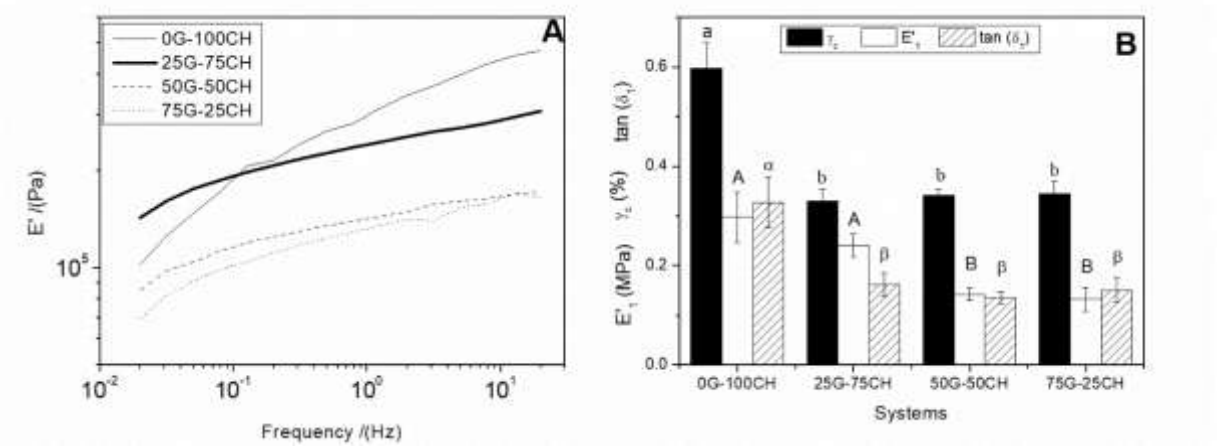

Figure 2 (A) Frequency scanning and (B) critical strain (γ_c), loss tangent and elastic module at 1 Hz ($\tan(\delta)_1$ y E'_1) of the scaffolds with different gelatin/chitosan (G/CH) proportions obtained by the standard methodology (A method). Differently labeled columns are significantly different (p <0.05).

3.2.2 Porosity

Porosity values for the different systems are shown in Table 2. It can be observed that porosity is above 96%, for all the systems, with slightly higher values for systems containing more CH.

3.3 Morphological Properties

The 0C–100CH system, being with better mechanical properties and porosity, was selected for carrying out morphological characterization through scanning electron microscopy where its microstructure and fiber distribution (porosity and pore interconnectivity) were studied. Figure 3 shows the micrography obtained for this system.

Figure 3A shows the surface of the scaffold with closed pores called the skin effect. On the other hand, good internal pore interconnectivity due to the fiber distribution, which benefits cell proliferation in these systems, was also observed (Figure 3B).

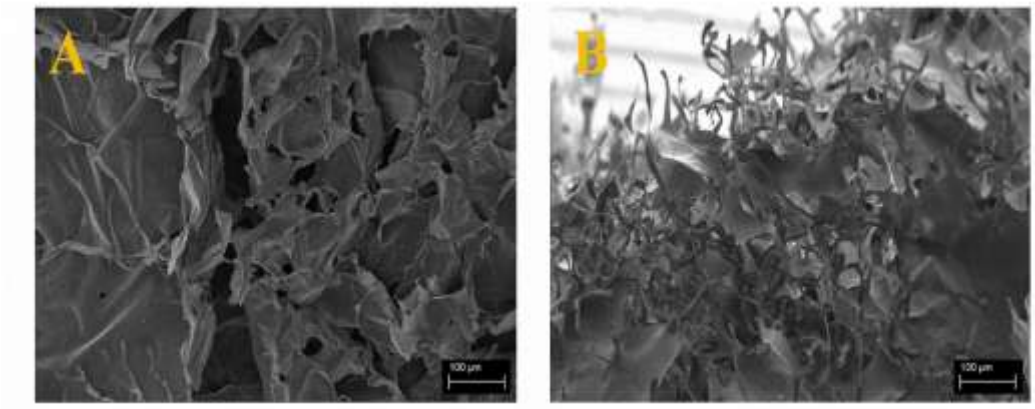

Figure 3 (A) Surface and (B) perpendicular view through SEM images of the system 0G–100CH produced by the reference protocol (method A).

4. Conclusions

Based on the results, porous protein-based matrices (scaffolds) were obtained through the phase separation technique (freezing and lyophilization of a solution) with appropriate mechanical and morphological properties for application in TE.

It was observed that the gelatin-chitosan ratio used for scaffold production leads to different mechanical properties and porosity. In general, higher chitosan content results in better scaffolds. On the other hand, the processing conditions also affect the properties of the scaffold. Heat treatment under agitation resulted in higher elastic modulus stability and critical strain due to the better structuration of the biopolymeric matrix chains.

However, regardless of the composition and manufacturing method, the obtained scaffolds are biopolymeric matrices with proper mechanical properties, porosity, and pore interconnectivity, parameters that favor cell growth and proliferation through the system.

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Author Contributions

MAG and JFRV carried out the experimental part of the research. VPP and MJR wrote the manuscript and provided background information. AR reviewed and supervised both the experimental part and the final manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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