RHEOLOGICAL CHARACTERIZATION OF HYDROGELS FORMULATED WITH ALGINATE OR PECTIN COMBINED WITH XANTHAN GUM AND GELATIN FOR BIOPRINTING APPLICATIONS

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ABSTRACT

Hydrogels comprising xanthan gum (X) for viscoelasticity, and either alginate (A) or pectin (P) for rapid bivalent ion crosslinking, were combined with gelatin (G) to enhance bioactivity for tissue engineering applications and 3D bioprinting. Rheological assessments of AXG and PXG formulations were conducted. Qualitative analysis of the ability of homogeneous filament formation and hydrogel flow capacity were performed by syringe extrusion followed by microscopic evaluation and tube inversion test. The assessment of crosslinking time was performed using 100 mM $CaCl_2$. The viscosity profile at 25 ºC with varying shear rates was analyzed using a rheometer. AXG and PXG hydrogels formed homogenous filaments upon extrusion and did not flow after up to 15 min of tube inversion. AXG fully crosslinked after 30 min exposure to $CaCl_2$, while PXG fully crosslinked after 55 min. AXG exhibited superior resistance to manipulation, possibly due to enhanced crosslinking facilitated by alginate chain functional groups. Post-sterilization, viscosity slightly decreased for both formulations, with sterile AXG showing 2.4x and 1.2x higher viscosity than PXG at 1 s^{-1} and 100 s^{-1}, respectively. Thus, this research offers promising tailored biomaterials for advancing the 3D bioprinting and tissue engineering fields.

Keywords: Hydrogel. Polysaccharides. 3D printing. Tissue Engineering. Rheology.

1 INTRODUCTION

3D bioprinting is an innovative technology that enables the precise fabrication of complex tissue structures by depositing bioinks layer by layer. This technique holds significant promise for various applications in tissue engineering, regenerative medicine, and drug discovery, as it allows for the creation of biomimetic tissues and organs that closely replicate the native physiological environment. The potential to engineer functional tissues and organs opens new avenues for personalized medicine, reducing the dependency on organ transplants and enabling more accurate disease models for drug testing. Hydrogels are hydrophilic polymer networks capable of retaining large amounts of water, and are among the most promising materials for use in 3D bioprinting. These materials can mimic the extracellular matrix, providing a supportive scaffold that promotes cell viability, proliferation, and differentiation. To be suitable for 3D bioprinting, hydrogels must exhibit specific characteristics, such as appropriate rheological properties to ensure smooth extrusion and precise deposition, structural stability to maintain shape fidelity post-printing, and ability to crosslink rapidly and effectively to provide mechanical integrity [1,2].

The materials used for the production of these hydrogels must be biocompatible, biodegradable, and possess adequate mechanical strength and rheology. The degradation products of these materials cannot be toxic and should be metabolized and excreted by the body at a rate compatible with tissue recovery from injury. In this context, natural polymers have been widely studied due to their biocompatibility, biodegradability, and similarity to the extracellular matrix [3]. Xanthan gum, pectin, alginate, and gelatin are among the biopolymers tested for this purpose.

Xanthan gum is a type of polysaccharide produced by the bacterium *Xanthomonas campestris*, showing attractive rheological properties for 3D bioprinting. The pseudoplasticity presented by the polymer when in solution allows it to flow easily through a syringe or printer nozzle, but it regains its viscosity when deposited, maintaining the shape of the printed material. This occurs because pseudoplastic materials have their viscosity reduced when a shear force is applied but can recover it when the shear is stopped [4,5].

Pectin is a polysaccharide found in the cell walls of plants and is also widely used in the food industry as a gelling agent. High-degree esterified pectins crosslink through hydrogen bonds and hydrophobic forces in low pH solutions with high sugar content. In contrast, low-degree esterified pectins can be crosslinked by divalent ions, such as calcium (Ca^{2+}), and free carboxyl groups [6].

Alginate, on the other hand, is a polysaccharide derived from brown algae and is one of the most studied materials for the composition of hydrogels for 3D bioprinting. This polymer can also be crosslinked by divalent ions, such as Ca^{2+}, exhibiting rapid gelation capacity in the presence of these ions. This ionic crosslinking mechanism is useful for increasing the stability and rigidity of the construct produced by 3D bioprinting [7].

Although it is possible to culture cells in hydrated polysaccharide matrices such as xanthan, pectin, and alginate, these compounds do not have molecular recognition sites that promote cell adhesion and migration, limiting the functionality of these materials. These sites consist of chemical groups present in small peptides that interact with receptors on the cell surface. There are many strategies for functionalizing biomaterials that lack the necessary adhesion sites, including mixing them with other biomaterials, such as gelatin. Gelatin, which is produced from collagen, contains peptides with the Arg-Gly-Asp (RGD) sequence, which binds to receptors on the cell surface and promote cell adhesion and proliferation [8].

Thus, in this work the combination of the biopolymers xanthan gum and pectin or alginate with the bioadhesive polypeptide gelatin was explored, aiming at the production of a hydrogel suitable for application in 3D bioprinting and tissue engineering.

2 MATERIAL & METHODS

Materials used to produce and crosslink the hydrogels: Alginate, pectin, gelatin, calcium chloride, and phosphate buffered saline, all from Sigma-Aldrich, as well as xanthan gum from CPKelco, and distilled water, were used throughout the work.

Preparation of the hydrogels: Xanthan gum, pectin, and alginate solutions were prepared in PBS. For complete dissolution of the polysaccharides and to obtain homogeneous solutions, the materials were subjected to mechanical stirring at 600 rpm for 30 min at room temperature. The pectin or alginate solution was mixed with the xanthan solution at a 2:1 mass ratio. In parallel, a gelatin solution was prepared in PBS under mechanical stirring at 37°C. The gelatin solution was then mixed with the alginate-xanthan or pectin-xanthan solution to obtain AXG and PXG hydrogel formulations. The resulting hydrogel, with a final concentration of 9% (w/v), was sterilized by autoclaving. The hydrogel was placed in syringes of up to 5 mL, centrifuged at 3000 rpm for 5 minutes to remove bubbles, and stored at 4°C. Samples were removed from refrigeration before the tests and all tests were performed at room temperature.

Qualitative analysis of flow behavior and crosslinking test: Qualitative analysis of the ability of homogeneous filament formation and hydrogel flow capacity were performed by syringe extrusion followed by microscopic evaluation and tube inversion test. The assessment of crosslinking time was performed using 100 mM $CaCl_2$.

Quantitative analyses of rheology - viscosity x shear rate measurements: The viscosity profile at 25°C with varying shear rates (0,1 s^{-1} to 3.000 s^{-1}) was analyzed using a rheometer. The tests were carried out in triplicates using an Anton-Paar MCR-10 modular compact rheometer, with a CP-50 cone measurement geometry, in collaboration with the laboratory of Professor Dr. Marcos Akira d'Ávila (FEM/UNICAMP).

3 RESULTS & DISCUSSION

In order to comprehensively characterize the formulations, a series of initial tests were conducted, encompassing tube inversion, filament extrusion, and gelation assessments. The tube inversion test provides valuable insights into the flow properties and structural stability of the hydrogel. By assessing whether the hydrogel maintains its shape when the container is inverted, it is possible to anticipate if the material will not deform or flow excessively during printing . This is essential for achieving accurate and precise deposition of the hydrogel layers, a critical issue for the fabrication of complex tissue structures with high fidelity. The tube inversion test with AXG and PXG formulations showed that the hydrogels did not flow when the tube was inverted for a duration of up to 15 min. This suggests that the hydrogels are capable of retaining the structural integrity post-deposition during the printing process, thereby ensuring a high degree of dimensional fidelity.

Filament extrusion testing evaluates the extrudability of the hydrogel through the printing nozzle. This test assesses the ability of the hydrogel to form uniform and continuous filaments, which are necessary for generating intricate tissue architectures with well-defined features. Consistent filament extrusion is essential for ensuring the reproducibility and reliability of the bioprinting process, ultimately influencing the quality and functionality of the printed tissue constructs. Both AXG and PXG hydrogels demonstrated the formation of uniformly homogeneous filaments upon extrusion through an 18G syringe needle, indicating consistent and controlled material flow characteristics conducive to printing applications.

Gelation assessments are vital for understanding the crosslinking time and mechanical properties of the hydrogel. Gelation refers to the formation of a three-dimensional network within the hydrogel matrix, which provides structural support and stability to the printed constructs. A gelation test was performed by exposing AXG and PXG formulations to a 100 mM $CaCl_2$ solution. Interestingly, the PXG formulation exhibited a delayed onset of crosslinking, which was observed only after 55 min of exposure to the $CaCl_2$ solution. Conversely, the AXG formulation achieved complete crosslinking within a shorter timeframe of 30 min under the same conditions. Notably, the AXG crosslinked formulation showcased superior resistance to manipulation, retaining structural integrity during handling, which can be attributed to the heightened crosslinking facilitated by functional groups present within the alginate chains.

The faster crosslinking of alginate chains compared to pectin when in a $CaCl_2$ solution can be attributed to their structural and chemical differences. Alginate contains guluronic acid residues, which are able to coordinate with calcium ions present in the calcium chloride solution. This interaction leads to the formation of ionic bridges between alginate chains, resulting in rapid gelation. On the other hand, pectin typically contains a lower proportion of carboxyl groups compared to alginate, reducing its ability to form strong ionic interactions with calcium ions. Additionally, the structure of pectin may hinder the accessibility of calcium ions to the carboxyl groups, further slowing down the crosslinking process. Therefore, the higher affinity and accessibility of alginate for calcium ions makes its crosslinking faster in $CaCl_2$ solution compared to pectin.

Rheology testing provides essential insights into the flow behavior and mechanical properties of the hydrogel, particularly its viscosity, which is a key determinant of printability. The viscosity of the hydrogel directly influences critical printing parameters such as extrusion pressure, nozzle size, printing speed, and layer thickness. An optimal viscosity ensures that the hydrogel flows smoothly through the printing nozzle, forming uniform filaments or droplets with controlled dimensions. This is essential for accurately reproducing complex tissue architectures and maintaining the desired structural integrity of the printed constructs. Before sterilization, rheology testing provides valuable insights into the intrinsic rheological properties of the hydrogel formulation. The post-sterilization rheological assessment ensures that the hydrogel maintains its printability and performance characteristics following sterilization, thus enabling consistent and reliable bioprinting outcomes. Viscosity was slightly reduced after sterilization for both formulations, and was 2.4x and 1.2x higher for sterile AXG when compared to PXG at 1 s^{-1} and 100 s^{-1}, respectively. This suggests that sterilization impacts hydrogel formulations differently, potentially due to variations in chemical composition or crosslinking density. Such changes in viscosity post-sterilization have implications for bioprinting and may require adjustments in printing parameters to ensure optimal flow characteristics. Understanding the effect of sterilization on viscosity is crucial for optimizing hydrogel formulations for bioprinting applications, facilitating the development of robust and reliable printing protocols.

4 CONCLUSION

This study comprehensively characterized hydrogel formulations for bioprinting, revealing their structural integrity, extrudability, gelation time, and rheological properties. Both AXG and PXG hydrogels exhibited suitable structural stability and controlled material flow, essential for precise tissue fabrication. Differences in crosslinking time highlighted AXG's superior resistance to manipulation. Additionally, post-sterilization viscosity changes underscored the importance of optimizing sterilization methods for maintaining rheological properties. These findings contribute to enhancing hydrogel formulations for bioprinting, facilitating the development of reliable tissue engineering protocols. Further bioprinting tests will be conducted to determine the most suitable parameters (flow, speed, needle size) for achieving high print quality for each formulation. Thus, this research offers promising tailored biomaterials for advancing the tissue engineering field.

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