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Development of Adaptable 3D-Bioprinted Scaffold for Tissue Regeneration

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3D printing is an additive manufacturing technique that can transform 3D virtual models into physical objects by layer-by-layer deposition of materials that can undergo rapid liquid-to-solid transformation after dispensing. Hydrogels are one of the most interesting and challenging classes of material systems that can be employed as inks, and hydrogel-based 3D printing has been exploited in tissue engineering and regenerative medicine. Here, hydrogel bio-inks using k-Carrageenan (kC) and poly(vinyl alcohol) (PVA) have been formulated with the objective of tuning the printability properties of bio-ink. The kC allows the systems to undergo rapid sol-to-gel transitions upon cooling from 60 °C and above to body temperature, while the need to introduce PVA is related to optimizing the viscosity of the ink solution to enable 3D printing with a continuous filament and to introduce interconnected porosity in the scaffold. γ-radiation-induced chemical modification of kC polymer powder is successfully used here as a mean to induce noticeable modifications in the polymer molecular weight distribution and polymer structure and to optimize the printing properties of bio-ink formulations. The study aims to develop hydrogel formulations with viscoelastic properties and sol-gel transitions suitable for use as bioinks for 3D printing scaffolds with adipose stem cell spheroids for cartilage or bone tissue reconstruction.

1. Introduction

Injuries, trauma, or diseases often result in tissue degeneration or damage within the human body. Traditionally, such conditions are treated through tissue transplantation, either within the same body (autograft) or between different individuals (allograft). However, these approaches have significant problems: autograft retrieval is expensive, painful, and may lead to donor site morbidity, including infections and hematoma; allografts face limitations due to a constrained supply and potential transmission of diseases or rejection by the recipient's immune system (Asadian et al., 2020).
Tissue engineering (TE) emerges as an innovative approach to overcome the limitations of organ transplantation and surgical reconstruction. It focuses on regenerating damaged tissues, rather than simply replacing them, using living cells, biocompatible and biodegradable porous scaffolds, and appropriate biochemical and physical factors to influence cell recruitment, viability and fate (Berthiaume et al., 2011).
Three-dimensional (3D) bioprinting technology is a highly customizable scaffold manufacturing technique that enables the fabrication of porous structures with complex geometries, while also allowing the simultaneous and precise placement of cells and growth factors. (Li et al., 2021).
Hydrogels, owing to their structural similarity to the extracellular matrices of biological tissues (Geckil et al., 2010), stand out as attractive scaffold materials. Their rheological properties and biocompatibility make them suitable as bioinks for the construction of layer-by-layer scaffolds with desired morphological and functional characteristics (Kafle et al., 2021). One promising biopolymer for the production of hydrogel scaffolds is kappa-Carrageenan (kC), a linear sulphated polysaccharide extracted from red algae (El-Fawal et al., 2017). Due to

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its backbone conformation, this hydrophilic polysaccharide resembles the naturally occurring glycosaminoglycans (GAGs), which are fundamental constituents of connective tissues. In particular, due to the presence of sulfate groups, it has similarities with chondroitin sulfate, the most abundant GAG primarily found in cartilage (Zhang et al., 2015). kC is soluble in water above 60 °C and forms stable gels upon cooling, especially in the presence of metal cations (Mangione et al., 2005) and can also be successfully modified with adhesion proteins to improve cell adhesion (Muscolino, et al., 2022*a*). However, challenges such as brittleness and limited compressive strength necessitate blending with more resilient polymers such as agar, gelatin, poly(N-vinyl pyrrolidone) (PVP), or poly(vinyl alcohol) (PVA) (Zhang et al., 2015). Introducing PVA optimizes the viscosity of the ink solution to enable 3D printing with a continuous filament and to introduce interconnected porosity into the scaffold (Muscolino et al., 2022*b*).
This work seeks to optimize a novel bio-ink for 3D printing by combining kC and PVA in varying weight ratios and tuning the printability properties. The study investigates the sol-to-gel transition of the aqueous PVA/kC solutions and examines the mechanical and morphological properties of the resulting hydrogels. A novel approach in scaffold development was implemented, exploiting γ-irradiation of kC powder to reduce the printing temperature. In fact, polysaccharides subjected to γ-irradiation in the solid state and in air undergo a dose-dependent reduction in molecular weight (Muscolino et al., 2024) as a result of random chain cleavage reactions, without the need for chemical reagents and thus subsequent purification. By using γ-irradiated kC powder, this research aims to redefine the thermal constraints associated with 3D bioprinting, offering a more favourable environment for cell survival and tissue regeneration.

2. Experimental Section

2.1 Materials

The k-Carrageenan (kC) polymer powder was supplied by Gelcarin ME 8625 FCM BioPolymer. Poly(vinyl alcohol) (PVA) powder, with a molecular weight of 146-186 kDa and a degree of hydrolysis greater than 99 %, was provided by Sigma-Aldrich. Deionized water was filtered through a membrane filter with a pore size of 0.22 µm and used for the preparation of the hydrogel blends.

2.2 γ-Irradiation

The kC powder was subjected to irradiation in air, using a Cs-137 γ source (MDS Nordion 1000 Elite, KTH Stockholm) with a dose rate of 0.12 Gy s^{-1} and total doses of 10 kGy and 40kGy, after being preconditioned in a dry atmosphere in a desiccator containing silica gel for one week.

2.3 Hydrogels preparation

kC solutions with a total concentration of 2 %w (kC2) or 4 %w (kC4) were prepared by weighing the polymer powder, either unirradiated (0 kGy) or irradiated at a dose of 10 kGy or 40 kGy, and mixing it with filtered water. The solubilization was conducted at 80 °C under continuous stirring until a homogeneous solution was obtained. For the preparation of PVA-kC blends, aqueous solution of PVA at 6 %w was prepared by weighing the polymer powder and mixing it with filtered water. The system was maintained at 90 °C under stirring conditions for 2 h. Then, the kC, either unirradiated (0 kGy) or irradiated at the dose of 10 kGy or 40 kGy, and water were weighed and added to the 6 %w PVA solution, all of which was placed in a water bath at 80 °C for 2 h, in order to obtain two solutions of PVA-kC: the first solution will be named PVA4kC2, where the weight percentage of PVA is 4 %w and the weight percentage of kC is 2 %w; the second solution will be named PVA2kC4, where the weight percentage of PVA is 2 %w and the weight percentage of kC is 4%w. Subsequently, once homogeneity was achieved, the solutions were allowed to cool naturally to room temperature, allowing sol-to-gel transition to form the hydrogels.

2.4 Rheological analysis in small-oscillatory conditions

The rheological analysis in small-oscillatory conditions was performed with a stress-controlled Rheometer AR G2 and HR 20 (TA Instruments), using a geometry made of a crosshatched steel plate of 20 mm diameter.

2.4.1 Temperature ramp measurement

The gelation behavior was studied by performing oscillatory rheometric measurements at small strains. The hydrogel solutions were poured directly onto the bottom plate of rheometer and were surrounded by silicon oil to prevent water loss. Prior to each measurement, the geometry was heated to 80 °C and the temperature of the rheometer plate was varied through a controlled system from 80 °C to 30 °C at 1 °C/min, and the frequency was set at 1 Hz. The gel point was determined by the intersection point of the storage modulus (G') and loss modulus (G'') curves.

2.4.2 Frequency sweep measurement

Frequency sweeps were performed at a fixed strain value, between 10^{-4} and 10^{-3}, chosen for each formulation from a strain sweep test at 1 Hz, to test the material within its linear viscoelastic region. The temperature was kept constant at 37 °C. The frequency was swept between 0.1 Hz and 10 Hz. The samples were placed on the rheometer bottom plate and, once the temperature ramp measurement was completed, the measurements started after 1 min of equalization time.

2.5 Scanning Electron Microscope Analysis

The morphology analysis of the samples was performed using a FEI Quanta 200 FEG Scanning Electron Microscope (SEM) at an accelerating voltage of 10 kV or 30 kV. Samples were frozen in liquid nitrogen and freeze-dried to remove water. The freeze-dried samples were cut to expose their cross-sectional surface, mounted on aluminum stubs using a layer of graphite adhesive and gold-coated by JFC-1300 sputter coater (JEOL) for 120 s at 30 mA before scanning.

3. Results and discussion

3.1 Effect of γ-Irradiated kC powder on sol-to-gel transition

In order to investigate the effect of the γ-irradiated kC powder on gelation, the elastic modulus (G') and the viscous modulus (G") of the formulations were analyzed by rheological analysis, when subjected to a temperature change. The G'-G" crossover was used as a rough estimate for the gel point of the system. The determination of the gel point requires a meticulous approach that involves performing a series of frequency sweeps at different temperatures, to determine at which temperature tan(δ) = G"/G' is independent of frequency (Winter, 1986); however, in some cases, the G'-G" crossover can provide a comparable approximation of the gelation temperature, denoted as T_{gel} (Kitazawa et al., 2014). The measurements were conducted on kC2 and kC4 to determine the impact of γ-irradiation on the mechanical properties of the polysaccharide alone, and on the PVA-kC blends to understand the effect on the bio-ink formulation. The measurements were carried out in the temperature range of 80 °C to 30 °C, at a constant cooling rate (1 °C/min). Figure 1 shows the results of the temperature ramps for kC2, kC4, PVA4kC2 and PVA2kC4 in three conditions: unirradiated (0 kGy), irradiated at a dose of 10 kGy and irradiated at a dose of 40 kGy. For all formulations, the crossover of the G' and G" curves occurs at lower temperature for systems with higher irradiation doses.

For instance, Figure 1A-B shows that in the unirradiated kC2 (kC2 0 kGy) and kC4 (kC4 0 kGy) samples the crossover occurs at around 45 °C and 70 °C, respectively. At an irradiation dose of 10 kGy, the crossover takes place at approximately 44 °C for kC2 (kC2 10 kGy) and 60 °C for kC4 (kC4 10 kGy). Finally, for an irradiation dose of 40 kGy, the crossover happens at about 42 °C for kC2 (kC2 40 kGy) and 55 °C for kC4 (kC4 40 kGy). γ-irradiation causes mainly chain degradation in polysaccharides (Muscolino et al., 2023) meaning that the number of entanglements between chains might be reduced upon radiation. Mobility of the polymer segments is hence possible even at lower temperature compared to the unirradiated system. This explains why the crossover temperature of irradiated systems at 40 kGy is lower than the one of irradiated systems at 10 kGy and even lower than the unirradiated one.

Figure 1C-D shows that for PVA4kC2 and PVA2kC4 the crossover temperature also decreases with an increase in irradiation dose. T_{gel} is valuable information for the design of temperature-sensitive materials, in particular for bio-inks to be used in 3D printing. PVA4kC2 exhibits a T_{gel} of 59 °C, and it was seen that the most stable printing conditions for this formulation were obtained using a printing temperature of 45 °C. (Muscolino et al., 2022*b*). This suggests that lowering the T_{gel} of these new bio-inks would enable a printing temperature compatible with cell viability, allowing 3D printing with adipose stem cell spheroids directly added to the formulation to be loaded in the ink cartridge.

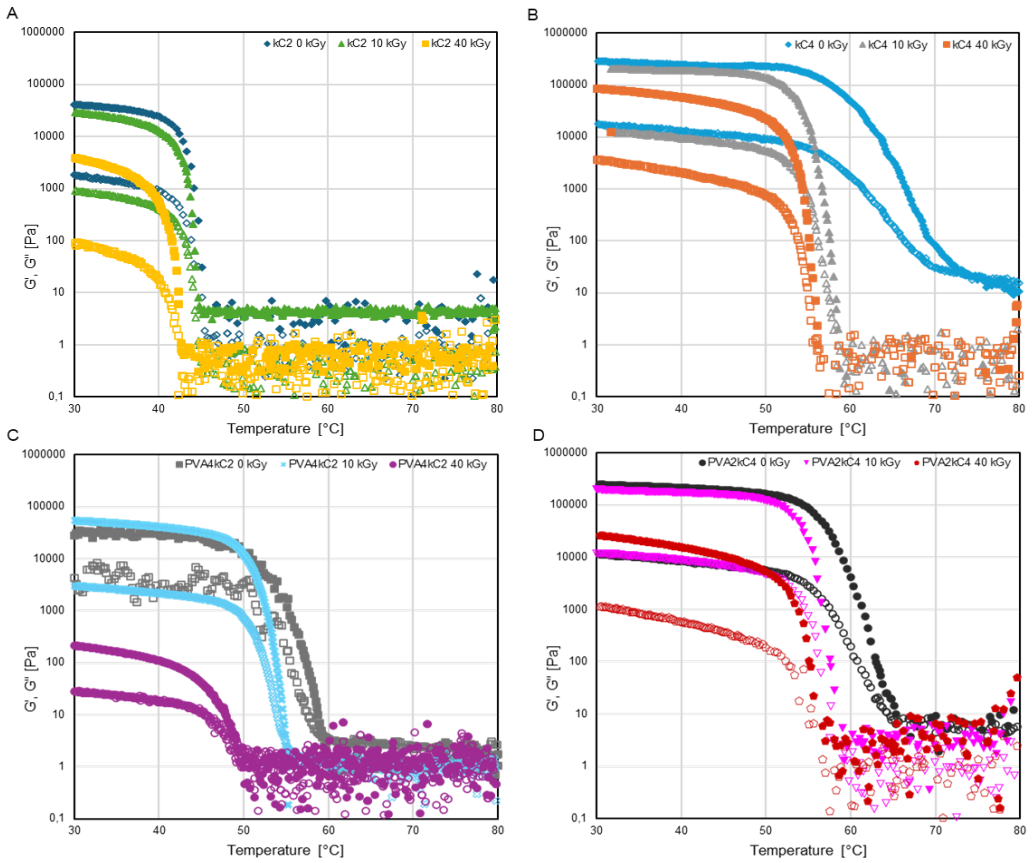

Figure 1. Storage modulus (G', full circle) and loss modulus (G'', empty circle) as function of the temperature (80 °C-30 °C) of kC2 (A), kC4 (B), PVA4kC2 (C) and PVA2kC4 (D), unirradiated (0 kGy), irradiated at 10 kGy dose and at 40 kGy dose. Experiments were carried out at cooling rate of 1 °C/min and frequency of 1 Hz.

3.2 Effect of γ-Irradiated kC powder on hydrogels properties

The mechanical properties of the systems, formulated with both unirradiated and irradiated kC powder, were tested to assess whether irradiation impairs the ability of the materials to function as supporting scaffold. The viscoelastic properties of hydrogels were evaluated by small-angle oscillatory strain sweep and frequency sweep tests. Strain sweep measurements were performed to explore the strain range of the linear viscoelastic region, at 1 Hz, and identify the optimal strain values to be set for the subsequent frequency sweep tests. The graphs in Figure 2 show G' and G'' as a function of frequency, for three different conditions: 0 kGy (unirradiated), 10 kGy and 40 kGy. The elastic modulus G' is a measure of the ability of the material to store energy in a fully reversible deformation, while the viscous modulus G'' is a measure of its resistance to flow, thus the energy the material can dissipate. All systems exhibit G' curves that are almost an order of magnitude higher than their corresponding G'' curves and mostly independent with frequency. This behavior is typically exhibited by fairly strong gels. Frequency sweep graphs of kC2 and kC4, prepared with unirradiated (0 kGy), irradiated at 10 kGy and irradiated at 40 kGy kC powder, indicate that γ-irradiation causes a decrease in G' and G'' moduli (Figure 2 A-B). In particular, irradiation at 40 kGy significantly detriments the mechanical properties. This is to be expected since irradiation should mainly cause degradation of the polysaccharide chain. The same can be observed for PVA2kC4 and PVA4kC2 at different doses of irradiation, but the effect of 10 kGy dose irradiation is less impacting. This means that a dose of 10 kGy can be used to lower T_{gel} without significantly affecting the mechanical properties.

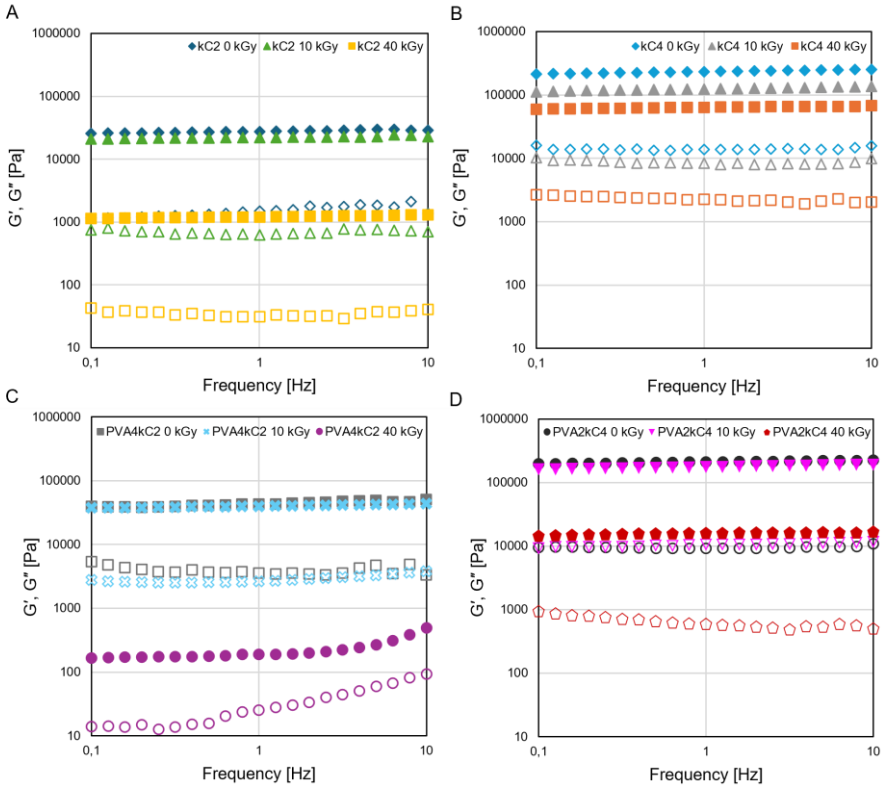

Figure 2. Storage modulus (G', full circle) and loss modulus (G", empty circle) as function of the frequency (1-10 Hz range) for kC2 (A), kC4 (B), PVA4kC2 (C) and PVA2kC4 (D), unirradiated (0 kGy), irradiated at 10 kGy dose and irradiated at 40 kGy dose.

From previous studies (Muscolino et al., 2022*b*), the content of PVA that also enables printing at reasonably low temperature, so as to perform cell co-printing, is significantly high (higher than kC). Among the formulations presented here, PVA2kC4 10 kGy represents the most interesting system because irradiated kC powder could allow an increase in kC content without increasing the sol-gel temperature or affecting the mechanical properties and a decrease in PVA content, which is a non-biodegradable polymer. Figure 3 shows the microstructures of PVA2kC4 0 kGy, PVA2kC4 10 kGy and PVA2kC4 40 kGy. The microstructure of PVA2kC4 0 kGy is characterized by pores with an irregular shape and wide size distribution (5-50 µm). PVA2kC4 10 kGy shows an increased density of the pores: a uniform distribution of small pores and larger cavities (10-100 µm), either empty or filled with the same porous material. This morphology is similar to that observed for PVA4kC2 (Muscolino et al., 2022*b*). In PVA2kC4 40 kGy, the cavities have disappeared and only a homogeneous structure of small pores is evident, as a sign of improved compatibility of kC 40 kGy with PVA. The degradation of chains in the material caused by γ-irradiation results in a change in microstructure. The breakage of polymer chains can cause their compaction and a reduction in pore size. This interconnected structure of uniform pores should constitute a favorable environment for cell proliferation, enabling rapid exchange of water, ions and nutrients.

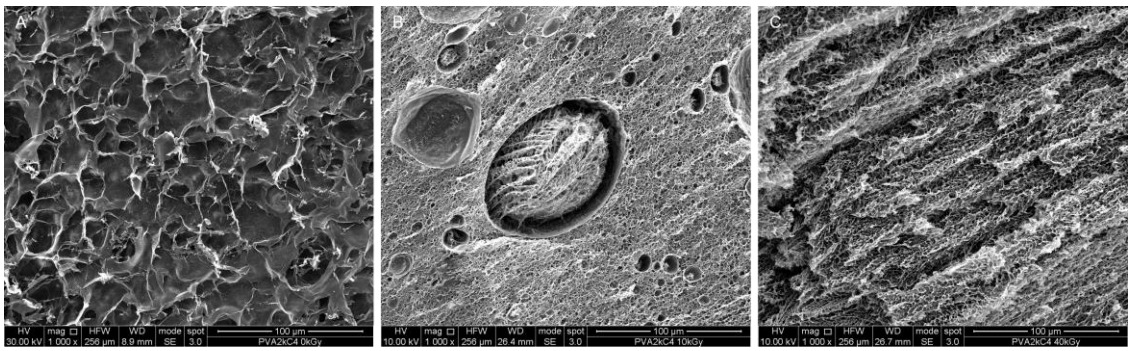

Figure 3. SEM micrographs of PVA2kC4 unirradiated (0 kGy) (A), irradiated at 10 kGy dose (B) and irradiated at 40 kGy dose (C).

4. Conclusions

The development of adaptable 3D-bioprinted scaffolds for tissue regeneration represents a promising avenue in tissue engineering and regenerative medicine. The study focused on characterizing the effects of γ-irradiation on kC powder within the polymer blend consisting of kC and PVA. Several tests, including strain sweep, frequency sweep and temperature sweep measurements, were conducted to evaluate the printability of the bio-ink solution. The results obtained showed a significant impact of irradiation on the printing temperature of the considered materials. In particular, rheological analysis showed that γ-irradiation of kC powder influenced the sol-gel transition temperature, with higher doses leading to lower gelation temperatures. This effect was observed in both kC alone and in PVA-kC blends. Furthermore, frequency sweep tests demonstrated that irradiation at lower doses can reduce the sol-gel transitions temperature, without significantly compromising the mechanical properties. Scanning electron microscope analysis revealed changes in the microstructure of hydrogels with irradiated kC powder, with an increase in pore density and uniformity observed at higher irradiation doses. The γ-irradiation has proven to be a decisive factor in reducing the temperature required for the printing process, with a more pronounced effect at higher doses, and the formulation consisting of PVA2kC4 10 kGy appears to be a promising material for bio-ink development. This finding suggests that irradiation could be a viable strategy for enhancing material processability during 3D printing, opening new perspectives for optimizing printing conditions and contributing to the advancement of additive manufacturing technologies.

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