

Photocrosslinkable *Kappa*-Carrageenan Hydrogels for Tissue Engineering Applications

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***Kappa* carrageenan (κ -CA) is a natural-origin polymer that closely mimics the glycosaminoglycan structure, one of the most important constituents of native tissues extracellular matrix. Previously, it has been shown that κ -CA can crosslink via ionic interactions rendering strong, but brittle hydrogels. In this study, we introduce photocrosslinkable methacrylate moieties on the κ -CA backbone to create physically and chemically crosslinked hydrogels highlighting their use in the context of tissue engineering. By varying the degree of methacrylation, the effect on hydrogel crosslinking was investigated in terms of hydration degree, dissolution profiles, morphological, mechanical, and rheological properties. Furthermore, the viability of fibroblast cells cultured inside the photocrosslinked hydrogels was investigated. The combination of chemical and physical crosslinking procedures enables the formation of hydrogels with highly versatile physical and chemical properties, while maintaining the viability of encapsulated cells. To our best knowledge, this is the first study reporting the synthesis of photocrosslinkable κ -CA with controllable compressive moduli, swelling ratios and pore size distributions. Moreover, by micromolding approaches, spatially controlled geometries and cell distribution patterns could be obtained, thus enabling the development of cell-material platforms that can be applied and tailored to a broad range of tissue engineering strategies.**

1. Introduction

Hydrogels are insoluble three-dimensional (3D) crosslinked networks of hydrophilic polymers, widely used as platforms in biomedical applications such as tissue engineered constructs,^[1–4]

drug delivery systems,^[5–7] cell-based therapies,^[8,9] wound dressings,^[10] and anti-adhesion materials.^[11,12] As the physical properties of hydrogels resemble the hydrated state of the native extracellular matrix (ECM),^[13] they exhibit high permeability towards oxygen, nutrients and other soluble factors,^[14] essential for sustaining cellular metabolism.^[15,16] The hydrogel networks can be fabricated by using physical or chemical crosslinking methods.^[17,18] Physical crosslinking is achieved through the formation of physical bonds between the different polymer chains. For example, ionically crosslinked gels are formed by the interactions between charged polymer chains and counterions.^[17] However, the uncontrollable exchange of ions in physical conditions reduces their applicability in the tissue engineering (TE) field.

On the other hand, through chemical crosslinking, stable covalent bonds between polymer chains are created.^[1,3] The formation of these permanent bonds is mediated by suitable crosslinking agents. Particularly, photocrosslinking, a type of chemical crosslinking, is performed in the presence of an ultraviolet (UV) light and a chemical photoinitiator (PI). Many photocrosslinkable materials are currently being investigated for TE applications, due to their processability and micromolding potential.^[19–21] Furthermore, it is possible to achieve a homogeneous distribution

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of cells and bioactive factors (e.g. proteins, growth factors) throughout the hydrogel matrix that can be easily delivered in situ.

Recently, natural origin polymers have attracted much interest due to their resemblance to the ECM, high chemical versatility, controlled degradability and interaction with biological systems. Amongst natural polymers, gellan gum,^[22] alginate,^[23] gelatin,^[24] hyaluronic acid,^[25] chitosan,^[26] chondroitin sulphate^[27] were modified in order to form hydrogels via photocrosslinking processes, while maintaining the viability of encapsulated cells. These natural polymers are also blended with other synthetic polymers to obtain unique property combinations for biomedical and biotechnological applications.^[28]

Carrageenans are a class of natural origin polymers, widely used as gelling, emulsifier, thickening, or stabilizing agents in pharmaceutical and food industry.^[29] Carrageenans are extracted from red seaweeds of the class *Rhodophyceae* and classified according to the presence and number of the sulphated groups on the repeating disaccharide units. Briefly, *kappa* (κ -) possesses one sulphated group, while *iota* (γ -) and *lambda* (λ -) possess two and three sulphate groups, respectively, per disaccharide unit.^[30] Their gelation occurs upon cooling under appropriate salt conditions by hydrogen bonds and ionic interactions, as both κ - and γ - undergo coil-helix conformational transition, rendering ionotropic and thermotropic gels.^[31] The gelation of κ -carrageenan (κ -CA) is enhanced mainly by potassium ions, forming firm, but brittle gels that dissolve when heated,^[32] while γ -carrageenan gelation is dependent on the presence of calcium ions, forming soft and elastic gels.^[17]

κ -CA has been proposed as a potential candidate for TE applications, due to its gelation properties, mechanical strength and its resemblance to natural glycosaminoglycans (GAGs).^[33] Furthermore, due to its inherent thixotropic behavior, κ -CA has been used as an injectable matrix to deliver macromolecules and cells for minimally invasive therapies.^[34] Although previous studies have shown encouraging results,^[35–37] there is still inadequate control over the swelling properties, degradation characteristic and mechanical properties of ionically crosslinked κ -CA hydrogels. This is mainly attributed to the uncontrollable exchange of monovalent ions with other positive ions from the surrounding physiological environment.^[22] Therefore, a modification of κ -CA that would enable the formation of stable crosslinked gels for cell encapsulation in which cell viability is preserved is a new challenge to be addressed. Previously, our work has shown successful chemical modification of natural polymers, leading towards the development of cell-laden platforms, based on photolithography and micromolding.^[25,23,38–40]

The primary aim of the current study is to design and develop a highly versatile micropatterned κ -CA hydrogel platform through dual-crosslinking mechanisms. We hypothesize that the crosslinking mechanism will influence the mechanical and viscoelastic performance of the developed system. Furthermore, cellular viability within modified κ -CA hydrogels, as well as the production of reactive oxygen species, will be evaluated as an assessment of their biocompatibility, potentiating their use in TE applications.

2. Results and Discussion

2.1. Synthesis of Methacrylated Kappa-Carrageenan

κ -CA is a linear, sulfated polysaccharide, composed of alternating 3,6-anhydro-D-galactose and β -D-galactose-4-sulphate repetitive units. It has recently been proposed for drug delivery applications^[41–43] and was shown to induce specific cellular responses, such as chondrogenesis of mesenchymal stem cells,^[36] or regeneration of an articular defect.^[44] κ -CA hydrogels can be formed via ionic crosslinking methods,^[45] however, these hydrogels present low stability in physiological settings, mainly due to the exchange of monovalent ions with the ones present in the surrounding environment. To overcome this drawback, we propose the chemical functionalization of κ -CA with methacrylate pendant groups, yielding photocrosslinkable hydrogels. The covalent bonds formed during crosslinking render high stability to the polymer network. Moreover, we hypothesized that the chemical modification along with the already present ionic character of κ -CA, will allow the formation of dual crosslinked hydrogels by combining chemical and physical crosslinking methods.

Methacrylated- κ -Carrageenan (MA- κ -CA), with various degrees of methacrylation, was synthesized by substituting the hydroxyl groups on κ -CA with methacrylate groups. The extent of substitution of hydroxyl groups was considered equivalent with the degree of methacrylation (Figure 1A), and was dependent on the volumes of methacrylic anhydride (MA) that were added to the reaction. Proton nuclear magnetic resonance (¹H NMR) spectroscopy of the modified polymer, confirmed the methacrylation of κ -CA by the presence of double peaks (vinyl) in the double bond region ($\delta = 5.5$ – 6 ppm) and one peak corresponding to the methyl ($-\text{CH}_3$) of the methacrylate group at $\delta = 1.9$ – 2 ppm (Figure 1B). To quantify the degree of methacrylation (DM), the degree of substitution of free hydroxyl groups present on the κ -CA backbone was evaluated by comparing the average integrated intensity of the methyl protons peak of the methacrylate group with the methylene groups present in the β -D-galactose (G6) of κ -CA. By adding 4%, 8% and 12% (v/v) MA to κ -CA during the synthesis, it was possible to create three different MA- κ -CA polymers with Low ($14.72 \pm 5.14\%$), Medium ($28.91 \pm 7.32\%$) and High ($37.11 \pm 7.41\%$) DM, respectively.

Furthermore, Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR) spectra revealed the appearance of the carbon-carbon double bond (C=C) at 1550 cm^{-1} , accompanied by the occurrence of the characteristic ester peak (C=O) at around 1680 – 1750 cm^{-1} , not present in κ -CA, thus confirming the methacrylation of κ -CA (Figure 1C). Moreover, all spectra showed an absorbance band at 1250 cm^{-1} , corresponding to the sulphate content characteristic of κ -CA. This indicates that the intrinsic sulphate group of κ -CA is also present in the modified formulations, thus it was not affected by the methacrylation reaction conditions. Furthermore, the zeta potential measurements confirmed the anionic potential of modified κ -CA and no significant difference in the overall charge was observed between non methacrylated and the methacrylated κ -CA (Zeta potential = -62.6 ± 1.3 mV and electrophoretic mobility = $-5.54 \pm 1.1\text{ }\mu\text{m}\cdot\text{cm}/\text{V}\cdot\text{s}$).

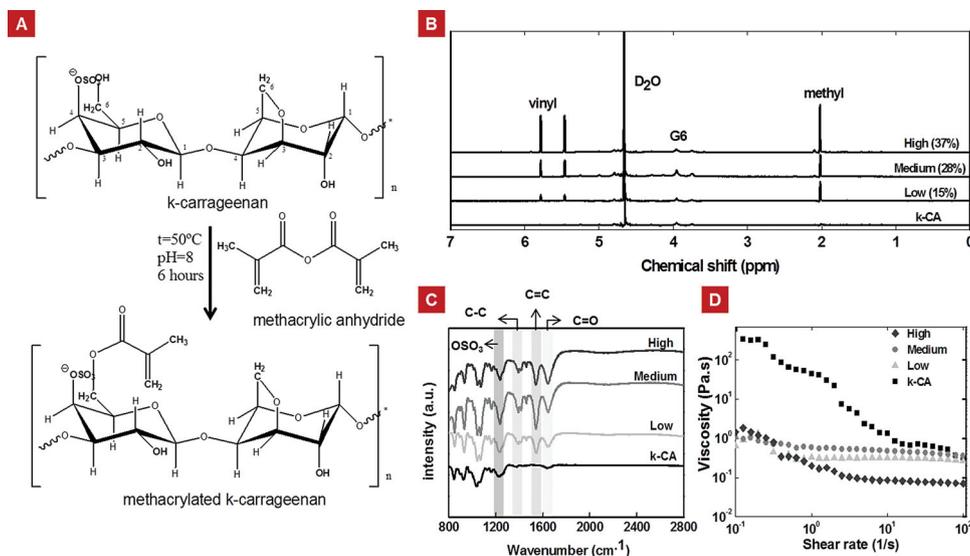


Figure 1. Characterization of methacrylated κ -carrageenan. (A) Schematic representation of the chemical modification of κ -CA. (B) ^1H NMR spectra ($T=50^\circ\text{C}$) of κ -CA, Low, Medium and High MA- κ -CA recorded in deuterated water (D_2O). The methylene protons of the β -galactose subunit of κ -CA (G6) were located at $\delta=3.89$ ppm and vinyl groups of the methacrylate ($=\text{CH}_2$) were found around $\delta=5.5\text{--}6$ ppm, while the methyl group ($-\text{CH}_3$) was located at $\delta=1.9\text{--}2$ ppm. The D_2O peak at $\delta=4.7$ ppm was used as internal reference. The degree of methacrylation (DM) was calculated as percentage (%) of the hydroxyl groups substitution with the methacrylate groups per each repeating unit. (C) ATR-FTIR spectra of modified κ -CA confirmed the grafting of methacrylate groups onto the polymer backbone. The peak appearing around 1550 cm^{-1} corresponds to the C=C bond of MA and is present in Low, Medium and High MA- κ -CA, while is absent in the non-modified counterpart. The C=O absorption band of the ester present around $1680\text{--}1750\text{ cm}^{-1}$ appears in all modified formulations. (D) The viscoelastic behavior of the polymer solutions shows that the methacrylation degree reduces the viscosity of the solutions highlighting the shear thinning behavior of the materials, suitable for injectable approaches. The data acquisition was performed at physiological temperature (37°C).

2.2. Viscoelastic Behavior of MA- κ -CA Solutions

κ -CA exhibits a reversible, temperature-sensitive gelation in salt-free conditions, through physical crosslinking. As a consequence, κ -CA chains undergo a sol-gel transition from random coil to coaxial double helix (soluble domains) configuration,^[46] as the temperature decreases. The conformational organization into double helices renders the formation of a 3D network maintained by the interaction of the polymeric chains with water through hydrogen bridges.^[47] The stability of this interaction allows the 3D network to undertake desired patterns, additionally conferring versatility to the κ -CA.^[48] Nevertheless, at room temperature, the κ -CA solutions are highly viscous and are difficult to manipulate resulting in poor processability.^[49] By increasing the temperature, the viscosity of κ -CA solutions dramatically decreased mainly due to the thermodynamic instability of the polymer chains. However, the range of temperature that must be employed ($50\text{--}70^\circ\text{C}$) is higher than the physiological temperature, which can compromise the viability of encapsulated cells or the activity of other incorporated bioactive components. Within this context, the viscosity of the precursor solutions of MA- κ -CA was evaluated. At physiological temperature, the modified κ -CA solution showed lower viscosities when compared with the non-modified κ -CA (Figure 1D). This change in viscosity can be attributed to the reduced interactions between side chains, ultimately leading to less double helix configurations.

Viscoelastic properties of κ -CA pre-polymer solutions show shear thinning behavior. At low shear rate (0.1 s^{-1}) the viscosity of κ -CA was around $500\text{ Pa}\cdot\text{s}$, while at higher shear rate (100 s^{-1}) the viscosity decreased to $1\text{ Pa}\cdot\text{s}$. After modifying κ -CA with methacrylation moieties, shear thinning behavior was suppressed. It was observed that MA- κ -CA requires lower applied shear rates to achieve the same viscosity values of κ -CA. This can be mainly attributed to the disruption of the ordered arrangement of double helix conformations by the presence of the methacrylate side chains.

2.3. MA- κ -CA Hydrogel Fabrication Via Different Crosslinking Mechanisms

κ -CA is capable of forming hydrogels through ionic interactions. Potassium salts are essential for κ -CA hydrogel formation, due to the interactions with the sulphate groups present on the backbone of κ -CA. These ionic interactions promote the condensation of the double helices into strong 3D networks. Moreover, the gelation, as well as the density of the ionic bonds in hydrogels, is strongly dependent on the presence, type and concentration of electrolytes. The introduction of methacrylate groups into the κ -CA backbone enables the formation of chemically crosslinked gels via UV exposure. Furthermore, as the anionic character of MA- κ -CA was not affected, it was still possible to form gels in the presence of K^+ salts. The

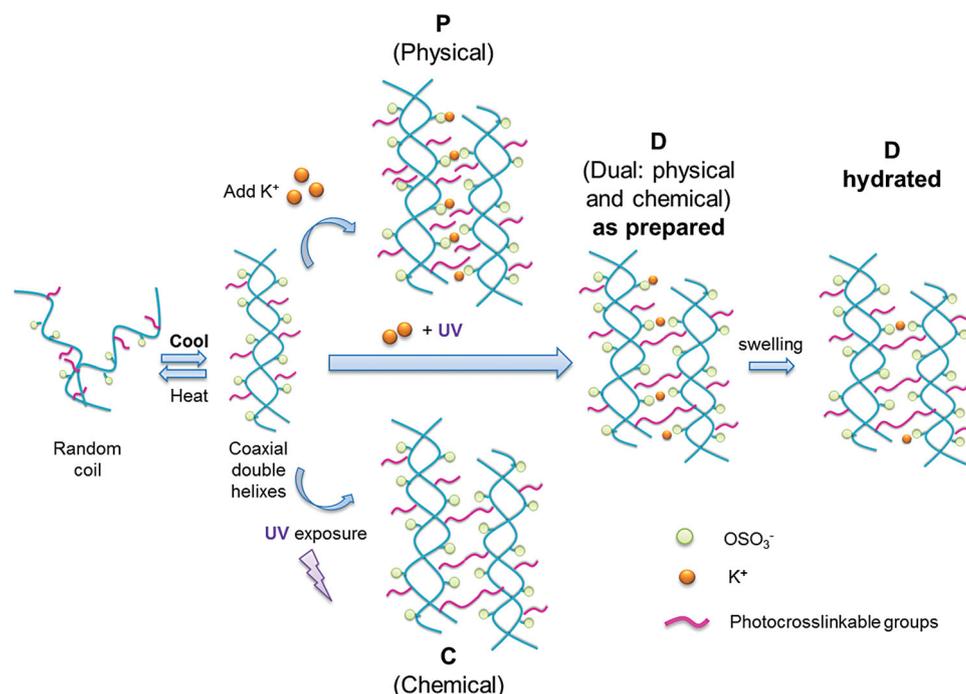


Figure 2. Proposed crosslinking mechanisms of methacrylated κ -CA. The thermoresponsive character allows the gelation of methacrylated κ -CA under salt-free conditions through the formation of physical bonds between the chains at low temperature. Due to the ionic nature of methacrylated κ -CA, the physical crosslinking (P) can occur in the presence of monovalent ions (K^+) forming stable gels. The pendant methacrylate groups can render the chemical crosslinking (C) by UV exposure. The association of the chemical and physical crosslinking approach allows the formation of dual crosslinked (D) hydrogels that are stable at physical conditions, as prepared or after reaching the swelling equilibrium.

presence of two functional groups (sulphate and methacrylate) can be independently used to tune physical properties as well as to encapsulate cells. As a consequence, the combination of two distinct crosslinking mechanisms enhances the versatility of the system, hence the range of potential application within the TE field. Also, it was noticed that the modified κ -CA showed a weaker response to temperature changes, which might be attributed to the additional methacrylate moieties that can hinder the complete packing of the double helix domains. The weak association between a polymer chains in double helix configuration due to functional groups grafted on the polymeric backbone was already reported for other natural origin polymers (e.g., gellan gum).^[50]

The schematic of the crosslinking of MA- κ -CA is depicted in **Figure 2**, and highlights the proposed crosslinking mechanisms employed in this study. Briefly, we developed physical (P) crosslinked hydrogels by crosslinking MA- κ -CA in the presence of K^+ ions; chemical (C) crosslinked hydrogels by photocrosslinking procedure, and a combination of the physical and chemical crosslinking mechanism, denominated as dual (D) crosslinked hydrogels.

2.4. Effect of Methacrylation Degree on Swelling Properties of the Hydrogels

In the context of TE, it is important to expose the polymeric networks to conditions that mimic *in vivo* environment. A proper evaluation of their behavior within this setup will allow

predicting the *in vivo* performance of the developed hydrogels. The swelling properties are a trademark of hydrogels properties, as these polymeric networks are able to retain water in various percentages dependent on their chemistry.^[4] Moreover, the swelling properties of polymeric networks are significantly influenced by water-material interactions. These affect the mass transport characteristics (nutrient and oxygen diffusion, waste disposal) and, consequently, their mechanical properties.^[51,52]

κ -CA is a hydrophilic polysaccharide that has shown significant properties changes when dissolved in aqueous solutions with different concentrations in ions.^[53] As mass swelling ratio can affect the overall features (shape and size) of a given patterned hydrogel the effect of the degree of methacrylation and crosslinking mechanism on the mass swelling ratio of the polymers hydrogels was evaluated under physiological conditions in Dulbecco's phosphate buffered saline (DPBS, Gibco, USA) and Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) at 37 °C. For this purpose, Low, Medium or High DM MA- κ -CA hydrogels were allowed to reach equilibrium over 24 hours in DPBS or DMEM at 37 °C, under dynamic conditions, to measure the mass swelling ratio (**Figure 3A,B**).

Holding the degree of methacrylation constant, a significant decrease in the mass swelling ratio ($***p < 0.001$) was observed, dependent on the crosslinking mechanism (**Figure 3A**). The chemical crosslinking mechanism allows the hydrogels to retain large volumes of solvent, due to the presence of covalent bonds/interactions. The chemically crosslinked network is highly flexible and allows the extension of the polymeric network without disrupting it. On the other hand, the short exposure to the

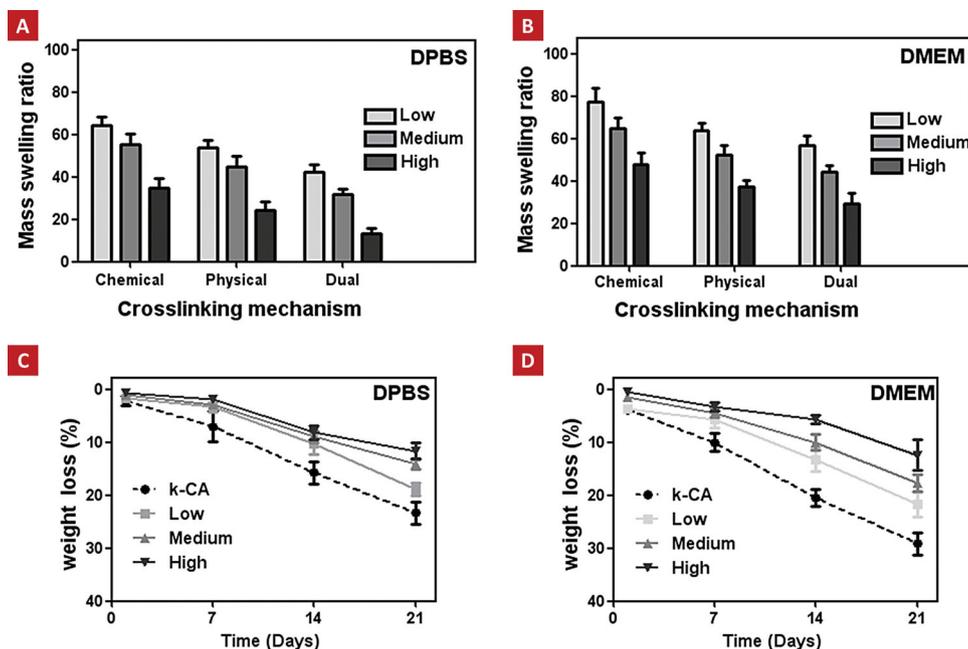


Figure 3. Swelling and stability of methacrylated κ -CA hydrogels in DPBS and DMEM. (A–B) The mass swelling ratios of 5% (wt/v) MA- κ -CA hydrogels in DPBS and culture medium, DMEM, show statistically significant differences. The dual crosslinking approach leads to the formation of hydrogels with low swelling properties. Representation of weight loss percentage vs time of dual crosslinked 5% MA- κ -CA hydrogels with different methacrylation degrees, immersed in DPBS (C) and (D)DMEM at 37 °C. All values are reported as corresponding to averages ($n = 3$) \pm standard deviation.

potassium chloride (KCl) treatment (physical crosslinking) of the MA- κ -CA hydrogels allows the diffusion of monovalent cations within the inner polymeric network, and the formation of strong ionic bonds between the chains. These bonds are less flexible; hence the ability of the polymer to retain the solvent is reduced by the physical crosslinking. By combining both crosslinking mechanisms it is possible to obtain hydrogels that significantly reduce their mass swelling ratio in DPBS.

Conversely, by maintaining the crosslinking mechanism constant, the mass swelling ratio is strongly dependent on the degree of methacrylation. The high MA- κ -CA exhibited significantly lower swelling ratios ($*p < 0.05$ when compared with the Medium MA- κ -CA, and $***p < 0.001$ when compared with Low MA- κ -CA) due to high crosslinking density. A similar behavior was observed for methacrylated alginate hydrogels where the decrease of dissociation degree occurred with the increase of the degree of methacrylation,^[24] as entanglement of covalent bonds reduces the degree of freedom of the network, hence reducing the ability to retain the solvent and enlarge its volume.

When immersed in cell culture media (DMEM) containing monovalent ions, divalent ions and proteins, hydrogels exhibit a similar swelling to DPBS (Figure 3B). However, the extent of the swelling is increased in DMEM when compared with DPBS. It is possible that the medium destabilizes the physically crosslinked network, by the substitution of the monovalent ions already present in the inner network of the hydrogel with the divalent ions or proteins present in the media. This allows the hydrogel to expand more and have higher swelling ratios.

Overall, these data suggest that efficient pattern fidelity can be achieved by increasing the degree of methacrylation and

applying both chemical and physical crosslinking procedures. Within these conditions the swelling ratios of the hydrogels are the lowest. Concluding, it is possible to produce hydrogels with adjustable swelling ratios.

2.5. Degradation/Dissolution Characteristic of the MA- κ -CA Hydrogels

The purpose of the dissolution studies was to evaluate the integrity and stability of the crosslinked MA- κ -CA hydrogels in physiological conditions. The dissolution of dual crosslinked hydrogels in DPBS (ions content) and DMEM (ions and protein content) was evaluated over a period of 21 days. During the dissolution period, κ -CA (physically crosslinked) showed to be less stable when compared with the dual crosslinked MA- κ -CA, in both dissolution media (Figure 3C,D). At day 21, 15% of the initial weight of physically cross-linked κ -CA was lost in DPBS, while in DMEM more than 25% of the initial weight was dissolved in the media. Contrary, it was observed that in the first 7 days of the experiment, all methacrylated formulations present a high stability and no evidence of disintegration/dissolution. At specific incubation time, it was noticed that the high methacrylation degree samples were less affected by the experimental conditions. This suggests that the increase in the degree of methacrylation renders hydrogels with strong bonding, upon chemical crosslinking, that cannot be disrupted by ionic exchange and interaction with proteins present in the culture media. Tuning the dissolution parameters is an interesting tool to use in order to address specific requirements of the TE objective.

Therefore, MA- κ -CA presents a versatile behavior in physiological conditions dependent on the degree of methacrylation. Envisioning TE applications, matching the extent to which the hydrogel loses its integrity with the rate at which native ECM is produced is a requirement.^[36,54] MA- κ -CA-based hydrogel possess the different degrees of degradation, dependent on the methacrylation degree.

2.6. Effect of Methacrylation Degree on Hydrogels Microstructure

The effect of degree of methacrylation on the dry microstructure of polymeric networks was evaluated using scanning electron microscopy. We used freeze-drying method to obtain dried polymeric structures. In this method, dual crosslinked hydrogels made from κ -CA with different methacrylation degree were subjected to a rapid snapshot cooling using liquid nitrogen and then the solvent (water) was removed by sublimation. Although this method cannot be used to visualize the original wet-polymeric network, can be used as a predictive tool of the effect of degree of methacrylation on the dried microstructure of MA- κ -CA, by evaluation of the pore size formed upon drying. The results indicated that κ -CA with high methacrylation degree results in a compact pore structure, with pore size of $18.5 \pm 5.3 \mu\text{m}$, while the medium and low methacrylated hydrogel showed interconnected structure with pore size of $33.2 \pm 8.2 \mu\text{m}$ and $48.8 \pm 10.4 \mu\text{m}$, respectively ($***p < 0.001$, Figure 4A-G). The decrease in pore size with an increase in methacrylation degree of dried structure can be attributed to high degree of crosslinking between polymeric chains. These observations in dried conditions are in agreement with the reported swelling behavior and rheological evaluation of the dual crosslinked hydrogels in fully hydrated conditions. In summary, hydrogels with low methacrylation degree have larger pores compared with hydrogels with high methacrylation degrees.

2.7. Effect of the Degree of the Methacrylation on 3D Network Stability and Mechanical Strength

2.7.1. Viscoelastic Behavior

The oscillatory shear experiments were performed to analyze the viscoelastic properties of the dual crosslinked hydrogels networks. The effect of the methacrylation degree on the viscoelastic properties was evaluated by monitoring the storage (G') and loss (G'') moduli. These parameters provide valuable information about the viscoelastic properties of hydrogels and have been taken into consideration in order to predict the stability of polymeric networks under shear forces. The ratio between the loss and storage moduli is defined as the loss tangent ($\tan \delta = G''/G'$), where δ is the loss angle. A stress sweep from 0.1 to 1000 Pa was performed at a constant frequency in order to evaluate the viscoelastic behavior of the dual crosslinked hydrogels.

Figure 5A shows the evolution of storage moduli with the increase of oscillatory stress. The non-modified κ -CA hydrogels formed only by physical crosslinking exhibits a shorter linear viscoelastic region, indicating a weak network that can

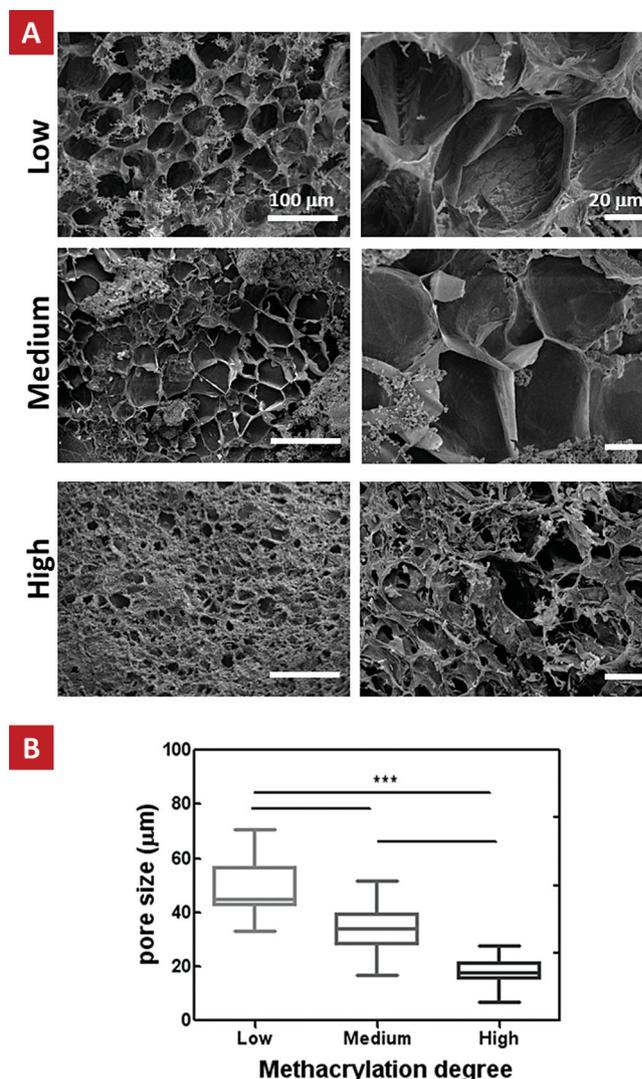


Figure 4. Microstructure of MA- κ -CA dual crosslinked dried hydrogels. (A) Representative SEM images of MA- κ -CA Low, Medium and High. (B) A significant decrease in pore size diameter with the increase in the methacrylation degree can be observed. All values are significantly different from each other ($n = 20$, ANOVA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Tukey post-hoc test).

be deformed at higher stress. However, the increase in the degree of methacrylation enlarges the viscoelastic region of the polymer network, suggesting that the chemical crosslinking via photocrosslinkable moieties renders highly stable networks. On the other hand, Figure 5B indicates the evolution of the viscous moduli with the increase of oscillatory stress. The low values of G'' show a trend similar to the G' , mainly dependent on the degree of methacrylation. At low stresses the G' values were higher than the ones corresponding to G'' , for all the samples. The loss angle (δ) of the crosslinked polymers also showed a dependence on the DM.

Figure 5C presents the crossover point where the value of $\tan \delta$ is 1 ($G'' = G'$). This critical point corresponds to the change of viscoelastic behavior from solid-like ($G' > G''$) to liquid-like

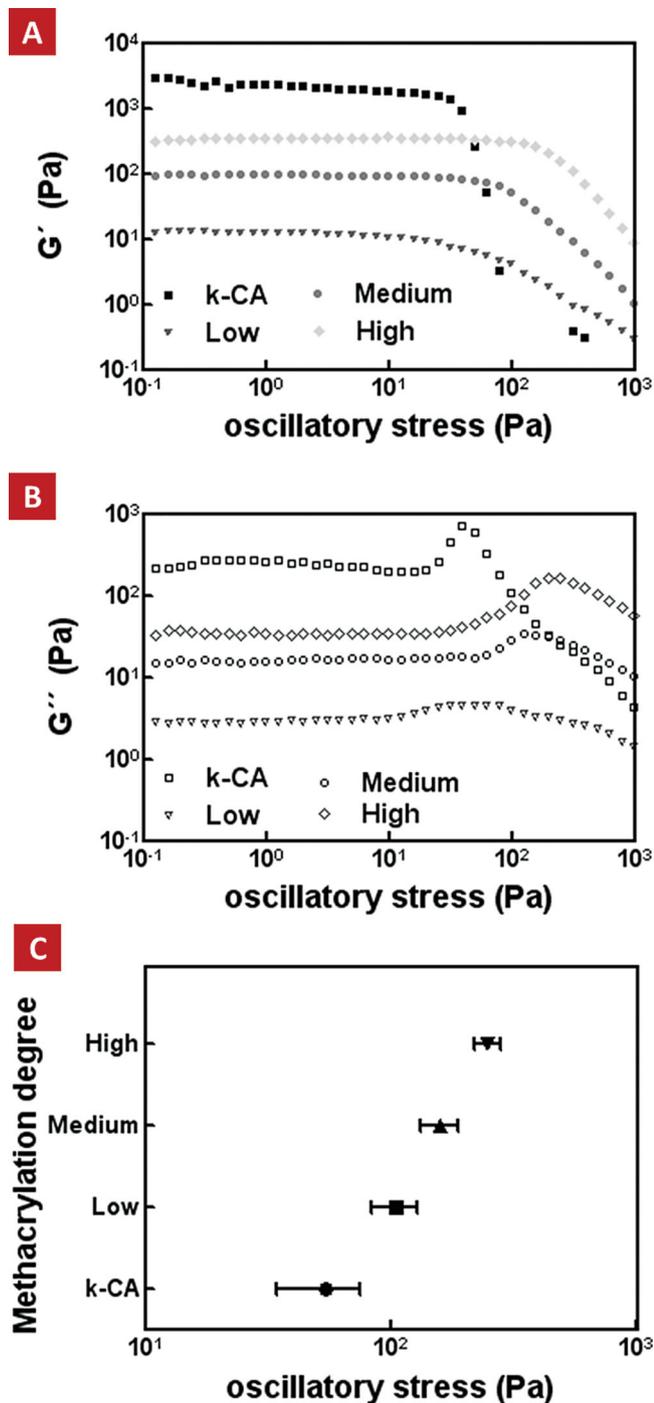


Figure 5. Viscoelastic properties, storage modulus (G') (A) and loss modulus (G'') (B) of swollen dual crosslinked hydrogels are strongly affected by the degree of methacrylation. (C) The increase of the methacrylation degree increases the photocrosslinkable units and the yielding of the internal network upon deformation, confirmed by the shifting to the right of the crossover point ($\tan \delta = 1$) between G' and G'' .

($G' < G''$) behavior. This transition is correlated to the collapse of polymeric network and consequently of the crosslinking bridges that bear the 3D network. The shift of the crossover point towards higher stresses indicates that hydrogel networks

are more stable for increased degree of methacrylation. This behavior was found to be similar to that showed by alginate containing both ionic and covalent links^[55] and polyethylene glycol nanocomposite hydrogels.^[18,56]

2.7.2. Compressive Properties

Considering hydrogels as cell carrier, modulator and delivery systems, it is important to address the stability of the polymeric network under stress-relaxation cycles, reproducing the repetitive force loads that native tissue are being exposed. The ionic crosslinking of κ -CA enables the formation of strong, but brittle network, not compatible with sustained loadings. In gels with ionic crosslinks, stress relaxes mainly through breakage and consecutive readjustment of the crosslinked bonds. In contrast, in gels with covalent crosslinks, like the methacrylate moieties, stress relaxes through the migration of water within the network,^[55] rendering a higher degree of stability within the network.

The mechanical properties of MA- κ -CA hydrogels were characterized by applying repetitive compression cycles on crosslinked hydrogels obtained from MA- κ -CA with different DM (Low, Medium and High). The influence of the applied crosslinking mechanisms and MA- κ -CA concentration on the mechanical performance of the developed hydrogels were also considered. Hydrated versus as prepared samples highlight the influence of the hydrated state on the mechanical properties of the hydrogels (Figure 6A,C). We observed that due to swelling, compressive moduli of all formulation decreased dramatically, however maintaining a certain trend dependent on methacrylation degree and on crosslinking mechanism (Figure 6B,D).

The increase of the degree of methacrylation augmented the stiffness of the dual crosslinked, due to higher crosslinking density, correlated with decreased mass swelling ratios. The increase in the density of photocrosslinkable units and the methacrylation degree lead to the formation of hydrogels with higher compressive moduli (Figure 6B). Dual crosslinked hydrogels depicted significantly higher compressive moduli when compared with the chemical crosslinking ($***p < 0.001$), and physical crosslinking ($**p < 0.01$), respectively.

Keeping the degree of methacrylation constant, chemical crosslinking enabled the formation of hydrogels with relatively lower compressive modulus ($**p < 0.01$) values, comparative to those fabricated only with the physical method. However, the combination of both physical and chemical crosslinking mechanisms, led to the development of hydrogels with significantly increased mechanical properties for all modified formulations ($**p < 0.01$). Even if the polymeric chain is occupied with methacrylate groups, the ionic interactions between chains are possible due to solvent transport throughout the network allowing the entrapment of monovalent ions between the chains and therefore an increase in elastic modulus values.

The mass swelling ratio plays an important role in the mechanical performance of all tested formulations, as the solvent molecules can easily penetrate the network loosening the chain-chain interactions. The κ -CA hydrogels present high compressive modulus due to the formation of double helix configurations that allow the chains aggregation. On the other hand, due to the increased percentage of photocrosslinkable

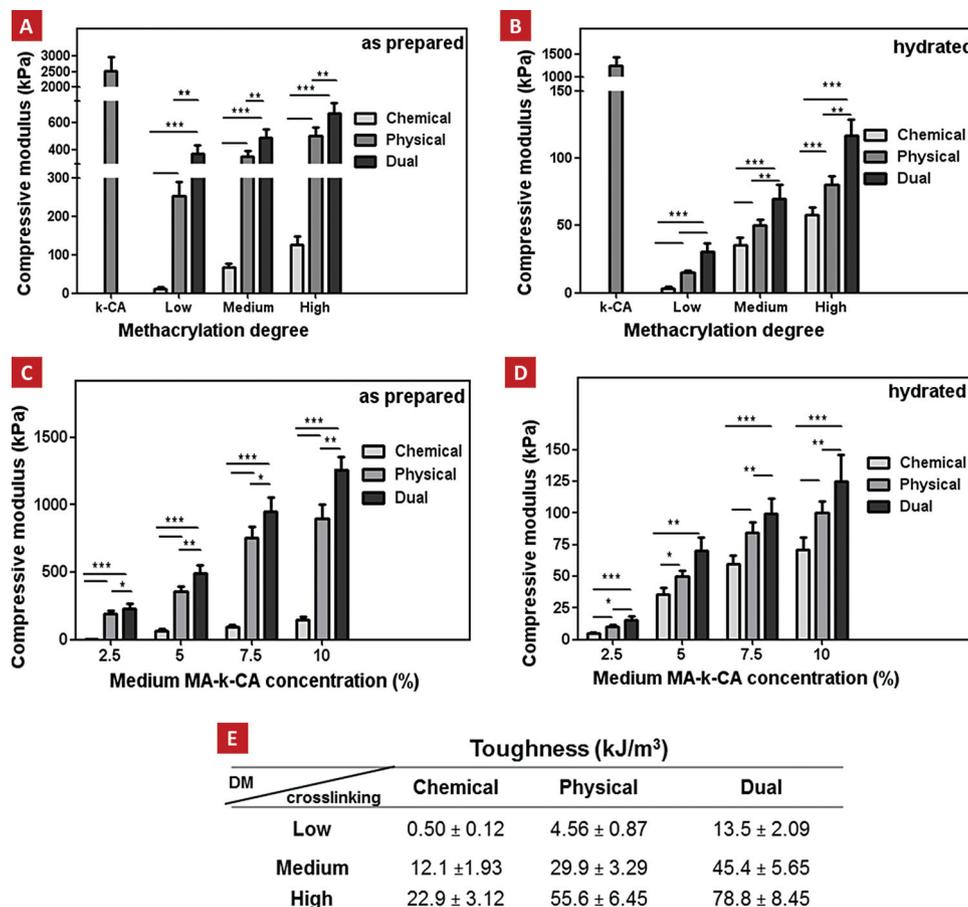


Figure 6. Mechanical properties of MA- κ -CA hydrogels obtained by different crosslinking mechanisms. The effect of the methacrylation degree, crosslinking mechanism, and hydration state over the compressive moduli of crosslinked hydrogels (containing 5 wt/v% polymer) was evaluated in (A) as prepared and (B) hydrated states. The effect of polymer concentration (medium methacrylation degree) over the values of compressive moduli for hydrogels in (C) as prepared and (D) hydrated state. (E) The increase of the methacrylation degree (DM), as well as the crosslinking mechanism applied, significantly increases the toughness of the hydrogels.

groups in MA- κ -CA polymer, the double helix conformation is compromised. However, the chemical crosslinking and the combination with physical procedure, leads to the formation of hydrogels with broad range of mechanical properties. The hydrogels resistance to network disruption is usually correlated with the density of the covalent bonds.^[57] The increase in the methacrylation degree renders the formation of tougher hydrogels (Figure 6C), due to the increase in the photocrosslinkable units, hence in the covalent bonds after crosslinking.

A similar trend was observed when the polymer concentration was increased. As mentioned above, it was also found a dramatic decrease of the compression modulus in hydrated samples, as compared to that registered for the samples tested as prepared. As explained previously, the affinity for water molecules allows the loss of monovalent ions and loosens the interactions between chains. At increased concentrations of Medium MA- κ -CA (2.5, 5, 7.5, and 10% w/v), the compressive modulus significantly ($*p < 0.05$) increased, due to the tight interactions between the chains as a consequence of crosslinking (Figure 6D,E).

Interestingly, with the increase of methacrylation degree the hydrogels acquired the ability to reversibly deform, without

loss of energy. This behavior was not noticed for physical crosslinked κ -CA (Figure 7A). The energy loss during deformation is proportional to the hysteresis of the loading curves for the two first cycles (Figure 7B). Using 50% strain level, the recovery of the gels after applying a loading cycle is ranging from 84.2 ± 6.5% for Low degree of methacrylation to 96.5 ± 4.1% for the High degree of methacrylation. Contrary, the κ -CA hydrogels, even possessing higher compressive moduli are not able to recover after the initial loading cycle. This behavior is supported by high energy loss (166.7 ± 17.23 kJ/m³ for the first cycle and 87.23 ± 10.34 kJ/m³ for the second cycle) and significantly ($***p < 0.001$) lower recovery percentage (47.5 ± 5.4%) when compared with the modified formulations (Figure 7C).

The elastic behavior of the dual crosslinked MA- κ -CA can represent an important asset if we consider the dynamic *in vivo* conditions. This data is in agreement with the rheology results that demonstrate the stability of the covalent network when applying increased stress rates. For a gel with ionic crosslinks, the stress relaxes as the crosslinks dissociate and reform elsewhere, so that the network undergoes plastic deformation, hence, the hydrogel cannot recover to its initial shape. On the

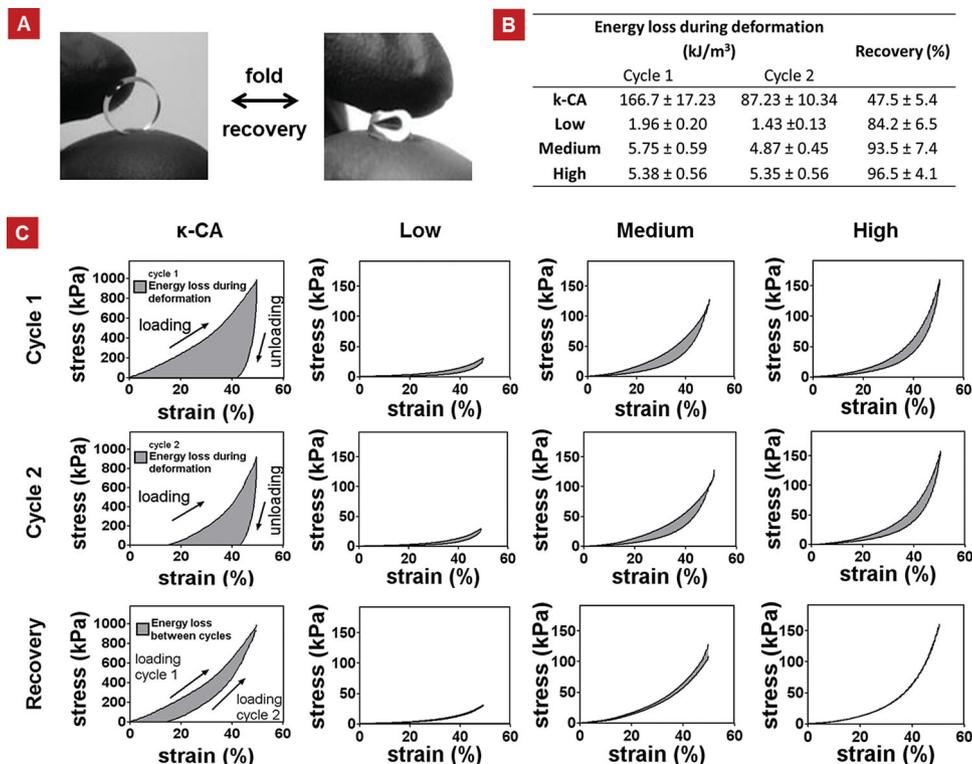


Figure 7. Compressive properties of methacrylated κ -CA hydrogels determined using unconfined cyclic compression. (A) Qualitative image showing the recovery of hydrogels undergoing deformation. This elastic behavior is not present for the non-modified κ -CA. (B) The effect of methacrylation degree on the loading and unloading cycle during the compression test is shown in hydrogels containing 5% (wt/v) polymer. The increase in the methacrylation degree increases the density of covalent bonds. As the hydrogels undergoes deformation, these bonds are flexible enough to allow the reorganization of the inner network, and therefore the recovery after deformation. Contrarily the κ -CA exhibits an irreversible plastic deformation as the polymer chain collapse after deformation. (C) The energy lost during the cycle can be calculated by determining the area between the curves. Polymer hydrogels are highly elastic and have negligible hysteresis. Increase in the methacrylation degree results in a decrease of energy lost during the cycle. For High formulation the recovery was about 95% compared with the Low formulation, which possessed a 72% potential for recovery.

contrary, introducing covalent crosslinks into the hydrogels network, by the photopolymerization process, the stress relaxes as water migrates out of the gel. Therefore, the network undergoes elastic deformation and can recover to the shape prior deformation.^[55]

Overall, the combination of physical and chemical crosslinking mechanisms resulted in the development of MA- κ -CA hydrogels with tunable mechanical properties, ranging from 3.0 ± 1.7 kPa to 107.0 ± 11.6 kPa. Hydrogels with biologically relevant mechanics can be obtained by means of physiologically compatible procedures, addressing a broad range of applications within the TE framework. Other methacrylate based hydrogels, e.g. gellan gum, displayed similar tunable mechanical properties features, by changing the degree of methacrylation, concentration of polymer and/or crosslinking mechanism.^[38]

2.8. 3D Cell Encapsulation in MA- κ -CA Hydrogels

2.8.1. Reactive Species Production (SO_x/NO_x)

Reactive oxygen species (ROS), such as superoxide anion (SO_x), and reactive nitrogen species (RNS), such as the nitric oxide

(NO_x), are generated as natural products of the cell respiratory metabolism and are produced in response to stress. It has been reported that apoptotic events are associated with direct or indirect activation of ROS.^[58] Therefore, the evaluation of the production of these species is of utmost importance to assess to which extent cells are exposed to stress conditions within the developed hydrogels.

The production of SO_x and NO_x, as indicators of oxidative and nitrosative stress in cells, was evaluated to determine to which extent the chemistry and formulation of hydrogels affected the encapsulated cells (**Figure 8A**). The initial SO_x and NO_x levels were attributed to stress exercised on cells as these were removed from cell culture flasks by trypsinization and consequent centrifugation and resuspension. However all of these procedures can perturb the levels of the oxides production, as cells react and adjust to the external stress. The encapsulation of cells in hydrogel networks increased the levels of SO_x and NO_x. As mentioned before, external stress generates a cascade of cellular events that can lead to increased levels of oxidative and nitrosative stress that can cause DNA damage, morphological transformations and cell membrane disruptions.^[59] However, the encapsulation in physical crosslinked κ -CA hydrogels does not significantly change the levels of SO_x/NO_x, when

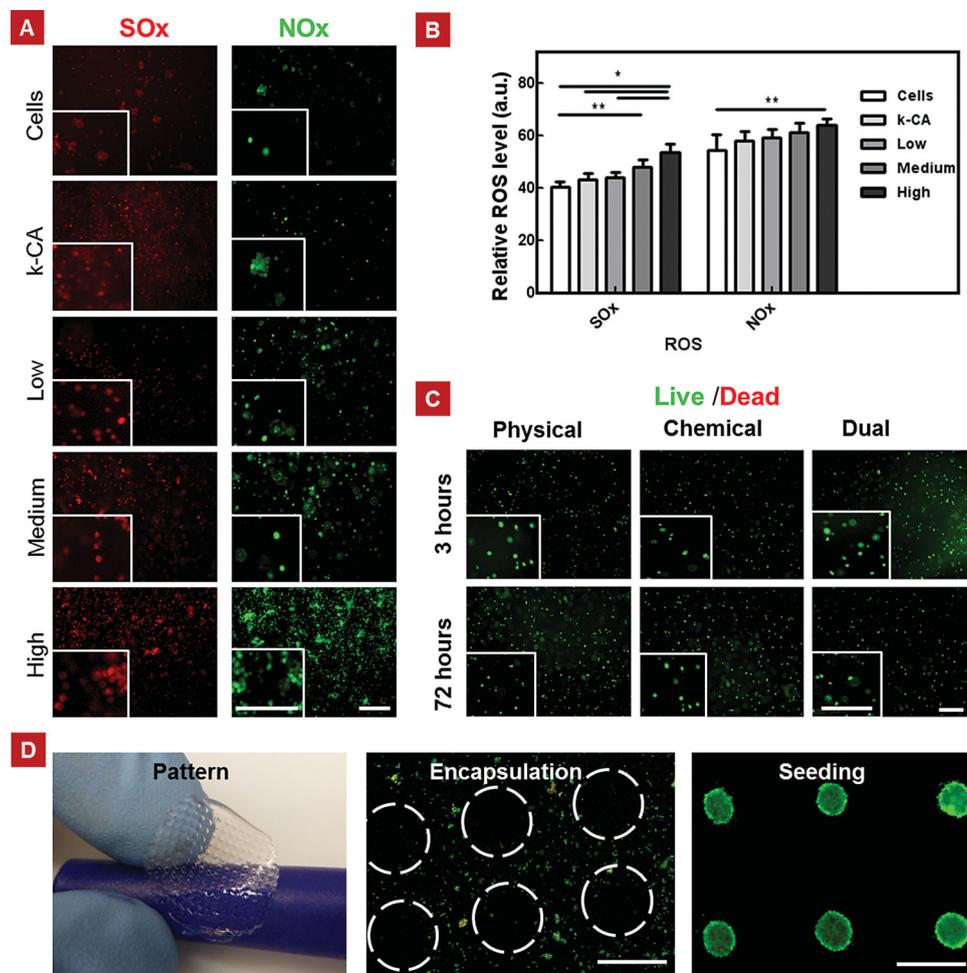


Figure 8. Cell encapsulation in MA- κ -CA hydrogels. (A) Production of intracellular superoxide (SOx) and nitric oxide (NOx) by NIH-3T3 cells after encapsulation in MA- κ -CA (5%, wt/v), 0.25% PI (wt/v), under UV exposure (40 seconds) and 10 min KCl (5%, wt/v) treatment. Trypsinized and encapsulated cells in κ -CA with KCl treatment were used as controls. The assay was carried out using dihydroethidium (DHE) and 4,5-diaminofluorescein diacetate (DAF-2DA) oxidation assay, respectively for SOx and NOx identification. (B) The quantification of the fluorescence intensity was assessed using NIH ImageJ software. Values are represented as average \pm SD, $n = 3$. Statistical differences ($*p < 0.05$, $**p < 0.01$) using one way-ANOVA followed by a Tukey post-test. (C) Representative fluorescence images of live (green) and dead (red) encapsulated NIH-3T3 cells in Medium MA- κ CA 5% obtained with different crosslinking mechanism, 3 h and 72 hours after encapsulation. (D) Patterns of different shapes and sizes can be obtained with the developed materials by micromolding. Cells can be encapsulated or seeded on a predefined pattern. Scale bar represent 100 μ m.

compared with cells alone, except for the High degree of methacrylation MA- κ -CA hydrogels.

On the other hand, the use of a photoinitiator, UV exposure and presence of methacrylate groups can seriously affect the cells integrity and viability, leading to poor functional response.^[60] We evaluated the production of SOx/NOx production levels in NIH-3T3 fibroblast cells as a function of crosslinking mechanism and degree of methacrylation. The photoinitiator and UV light did not show any significant influence on both residual production of SOx and NOx. However, high degrees of methacrylation caused a significant increase on the local levels of reactive species.

2.8.2. 3D Cell-Laden Hydrogels

Previous studies have showed that κ -CA hydrogels sustain the viability and enable proliferation of different cell types.^[36,54]

Herein, we evaluated the NIH-3T3 fibroblast cells viability encapsulated within photocrosslinked MA- κ -CA hydrogels by using a standard Live/Dead assay (Figure 8B). Cell viability was evaluated 3 hours after encapsulation, in order to assess the immediate effect of the applied crosslinking mechanism. The UV exposure time, photoinitiator concentration and methacrylate groups density of the MA- κ -CA formulations, showed no significant effect over the encapsulated cells viability. A long-term effect of the mentioned factors was assessed after 3 days of culture. Again, the viability of the encapsulated cells was not significantly affected.

The feasibility of the modified κ -CA to be used as materials for micromolding approaches was evaluated by creating MA- κ -CA patterns using polydimethylsiloxane (PDMS) templates. Cell-laden hydrogels of different shapes and sizes and easily manipulated were obtained (Figure 8C). It was noticed that the most reliable and consistent patterns were achieved

using High MA- κ -CA, mainly due to their low swelling behavior. Overall, the consecutive crosslinking procedures (UV and KCl) and the presence of photoinitiator showed no significant effect on the viability of the cells. These results strengthen the potential of MA- κ -CA hydrogels to be used as cell carriers within a spatially controlled distribution.

Apart from NIH-3T3 fibroblast cells, we also encapsulated MC3T3 E1-4 preosteoblast cell line, as well as human mesenchymal stem cells (hMSCs), within the MA- κ -CA hydrogels (data not shown). Preliminary results indicated that cells encapsulated for long time periods (up to 21 days), possess high viability (~75%) within hydrogels. This indicates that dual crosslinked MA- κ -CA systems can exhibit a high potential for a range of biomedical applications.

3. Conclusions

Methacrylated κ -Carrageenan was synthesized by reacting κ -Carrageenan with various amount of methacrylic anhydride, rendering the development of MA- κ -CA with different degrees of methacrylation. To our best knowledge, this is the first study introducing a photocrosslinked κ -CA with controllable elastic moduli, swelling ratios and pore size distributions. These physical properties can be easily tailored by varying the degree of methacrylation. Moreover, the combination of physical and chemical crosslinking procedure led to the formation of hydrogels with versatile mechanical and physical performance, while permitting maintenance of viable encapsulated cells. By micromolding approaches, spatially controlled geometries and cell distribution patterns can be obtained thus enabling the development of cell-material platforms that can be applied and tailored to specific functionalities in tissue engineering applications.

4. Experimental Section

Synthesis of methacrylated- κ -Carrageenan: MA- κ -CA was synthesized by reacting κ -CA with MA. Briefly, κ -CA was mixed at 1% (wt/v) into deionized water (diH₂O) at 50 °C until the polymer was fully dissolved. To this solution, MA was added and allowed to react for 6 hours at 50 °C. The pH (8.0) of the reaction was periodically adjusted with 5.0 M NaOH (Sigma, Germany) solution in diH₂O. The modified κ -CA solutions were dialyzed against diH₂O using 12-14 kDa cutoff dialysis tubing (Fisher Scientific, Cambridge, MA, USA) for 3 days at 4 °C to remove excess of unreacted MA. Purified MA- κ -CA solutions were frozen at -80 °C and then lyophilized. The obtained powder was stored at a -20 °C, protected from light until further use. To modify the degree of methacrylation (DM), the volumes of MA added in the methacrylation reaction (i.e., 4% (v/v) - Low, 8% (v/v) - Medium and 12% (v/v) - High (v/v)) were varied.

Characterization of methacrylated- κ -Carrageenan: The chemical modification of κ -CA was quantified by ¹H NMR spectroscopy. The ¹H NMR spectra of κ -CA and MA- κ -CA were collected in deuterated water (D₂O) at 50 °C, at a frequency of 500 MHz on a Varian INOVA NMR spectrometer with a single axis gradient inverse probe. All spectra were analyzed using 1D NMR Processor software (ACD/Labs 12.0). Phase correction was applied to obtain accurate absorptive peaks, followed by a baseline correction to obtain the integrals of the peaks of interest. The obtained chemical shifts were normalized against the protons of the methylene group of the D-galactose units (G) as an internal standard, which is present at $\delta = 3.89$ ppm.^[41] The DM was calculated referring to the peaks at $\delta = 1.9$ –2 ppm (methyl) and $\delta = 5.5$ –6 ppm (double bond

region) as percentage (%) of the free hydroxyl groups (-OH) substituted with methacrylate groups. FTIR-ATR analysis was performed on a Bruker Alpha FTIR spectrometer (Bruker Optics, MA, USA). The spectra were recorded at a resolution of 4 cm⁻¹ and the results are shown as an average of 24 scans. The zeta potential of the modified κ -CA was measured by laser Doppler anemometry using a Malvern Zeta Sizer 300HS (Malvern Instruments, UK). Each sample was diluted in water at a concentration of 0.1% (wt/v). Each analysis was performed at 25 °C, and lasted 60 seconds.

Preparation of dual crosslinked MA- κ -CA hydrogels: Freeze dried MA- κ -CA macromer with different DM, as well as non-modified κ -CA were added to a PI solution consisting of 0.25% (wt/v) 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals), in diH₂O, at 80 °C until complete dissolution. Physically crosslinked MA- κ -CA hydrogels were obtained by pouring the polymer solution into PDMS circular molds (8 cm diameter) followed by gently adding a solution of 5% (wt/v) of KCl (Sigma, Germany) to initiate the crosslinking. After 10 min of gentle shaking, samples were removed from the molds and washed in DPBS (Gibco, USA) in order to remove the salt residues. The chemically crosslinked hydrogels were obtained by pipetting 100 μ L of polymer solution between a Teflon substrate and a glass coverslip separated by a 1 mm spacer followed by UV light exposure at 6.9 mW/cm² (320–480 nm, EXFO OmniCure S2000, Ontario, Canada) for 40 seconds. To obtain dual crosslinked hydrogels, the chemically crosslinked samples were immersed in a 5% (wt/v) KCl coagulation bath for 10 min and rinsed with DPBS.

Swelling behavior and in vitro dissolution properties: The effect of the degree of methacrylation and crosslinking mechanisms on the swelling behavior and stability of the hydrogels was determined by evaluation of the hydration kinetics and dissolution behavior. Briefly, MA- κ -CA hydrogels obtained with different crosslinking procedures were lyophilized and weighted (initial dry weight, W_{DI}) before being transferred to 1.5 mL eppendorfs and soaked in 1 mL of DPBS and DMEM (Gibco, USA) at physiological temperature (37 °C), under constant shaking (60 rpm).

To assess the swelling properties, hydrogels ($n = 3$) were allowed to reach equilibrium in the swelling solution and weighted to determine the wet weight (M_w) after being blotted off with a KimWipe paper to remove the excess liquid from the samples surface. Then the samples were lyophilized to determine their final dry weight (W_{DF}). The mass swelling ratio was defined as the weight ratio of the liquid (DPBS or DMEM) uptake (M_w) to the weight of the dried hydrogel (M_{DF}), according to Equation 1.

$$\text{Mass swelling ratio} = \frac{M_w}{M_{DF}} \quad (1)$$

The dissolution degree was calculated by measuring the mass loss of the sample (Equation 2).

$$\text{Dissolution degree} = \frac{M_{DF}}{M_{DI}} \times 100 \quad (2)$$

Scanning electron microscopy (SEM) of dried dual crosslinked MA- κ -CA hydrogels: The microstructure of hydrogels was evaluated using a Scanning Electron Microscopy, SEM (JSM 5600LV, JEOL USA Inc., Peabody, MA, USA) at an acceleration Voltage of 5 kV and a working distance of 5–10 mm. First, hydrogel samples were plunged in liquid nitrogen slush, transferred to eppendorfs and freeze dried for 24 hours. The dry samples were fractured and then mounted on samples holders using double-sided carbon tape. The coating of the samples was performed with gold and palladium using a Hummer 6.2 sputter coated (Ladd Research, Williston, VT, USA). The quantification of the pore size distribution was performed using NIH ImageJ software, based on the SEM pictures.

Viscoelastic properties of MA- κ -CA hydrogels: The effect of methacrylation degree on the viscoelastic behavior of MA- κ -CA was determined using an AR2000 stress controlled rheometer (TA instruments, New Castle, DE,

USA) equipped with 20 mm flat geometry and the gap of 500 μm . Flow experiment was performed to evaluate the viscosity of polymer solution at 37 °C (shear rate varying from 0.01 to 100 s^{-1}). For dual crosslinked hydrogels, oscillatory stress sweep was applied between 0.1 to 1000 Pa at 37 °C and at a frequency of 0.1 Hz. The G' , as well as the G'' of the swollen samples, was measured using a gap of 500 μm and a 20 mm parallel plate geometry. A solvent (DPBS) trap was used in order to minimize the drying of the swollen hydrogels undergoing analysis.

Uniaxial compression testing: To assess the effect of the degree of methacrylation, crosslinking mechanism and polymer concentration over the mechanical behavior of the MA- κ -CA crosslinked hydrogels, cyclic compressive tests were performed using an Instron 5542 mechanical machine (Instron, Norwood, MA, USA). κ -CA and MA- κ -CA hydrogel discs (1 mm thick, 8 mm in diameter, $n = 6$) were tested at a rate of 10% strain/min (0.1 mm/min) until 50% of strain, for 5 complete compression-recovery cycles. Samples obtained immediately after crosslinking (as prepared samples) and samples that were allowed to swell in DPBS for 24 hours before testing (hydrated samples) were analyzed. The compressive modulus was defined as the slope of the linear region of the strain/stress curve, corresponding to 5–15% strain.

Reactive species production: Intracellular production of SO_x and NO_x was evaluated respectively using dihydroethidium (DHE, Molecular Probes, Eugene, OR, USA) and 4,5-diaminofluorescein diacetate (DAF-2DA, Calbiochem, San Diego, CA, USA) oxidation assays. NIH 3T3 fibroblasts were cultured in basal DMEM, containing with 10% of heat-inactivated fetal bovine serum (HiFBS, Gibco, USA) and 1% Pen/Strep (penicillin/streptomycin, 100U/100 $\mu\text{g}/\text{mL}$, Gibco, USA), at 37 °C, in a humidified atmosphere with 5% of CO₂. When reached 70% confluency, cells were trypsinized (0.05% Trypsin/EDTA, Gibco, USA) and further centrifuged at 1200 rpm, for 5 min. Cells (2×10^5 cells) were preincubated with DHE (25 μM) for 10 min and DAF-2DA (5 μM) for 30 min, at 37 °C, respectively for SO_x and NO_x detection. Cells were then washed with, centrifuged at 1200 rpm, for 5 min, and resuspended in 5% (wt/v) MA- κ -CA solution with different DM (Medium, Low, High) in 0.25% PI (wt/v) and 1.5% κ -CA (wt/v). The methacrylated polymers containing cells were UV crosslinked for 40 seconds and the obtained hydrogels were immersed in a bath of 5% KCl for 10 min for further crosslinking. The obtained hydrogels were washed with DPBS and incubated at 37 °C in phenol-red free DMEM (Gibco, USA) for 2 and 1 hours, respectively, for SO_x and NO_x detection. Preincubated cells, mounted on glass slides using Fluoromount mounting media (Sigma, Germany), were used as controls. All samples were examined under an inverted fluorescence microscope (Nikon, Eclipse TE 2000U, Japan). The quantification of the intensity of fluorescence was analyzed using NIH ImageJ software, considering the intensity of fluorescence per single cell for each of the evaluated conditions.

3D Cell encapsulation in MA- κ -CA microfabricated patterns: The effect of the degree of methacrylation, as well as the photocrosslinking conditions, on the cells viability was evaluated by encapsulating NIH-3T3 fibroblasts within gels prepared by applying both physical and chemical crosslinking. Cells were resuspended in 5% (wt/v) MA- κ -CA polymer containing 0.25% (wt/v) PI at a density of 2×10^6 cells/mL polymer solution. The cells suspension was poured onto Teflon sheet and using coverslip spacers, cell-laden hydrogels of 450 μm were formed after UV light exposure and further crosslink in a coagulation bath, as described previously. Afterwards, hydrogels were rinsed with DPBS and placed into 6-well cell culture plates. To form circular patterns, PDMS molds were used while crosslinking (100 μm diameter \times 300 μm depth \times 450 μm height). The viability of the encapsulated cells within the hydrogels was evaluated at 3 and 72 hours of culture with a Live/Dead (Invitrogen, Carlsbad, CA, USA) assay. Briefly, cells were incubated with calcein AM/ethidium homodimer-1 in phenol red free DMEM for 40 min. Fluorescence images were taken as previously mentioned.

Statistical analysis: All data values are presented as mean \pm standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5.00 software (San Diego, USA). Statistical significances ($*p < 0.05$, $**p < 0.01$ and $***p < 0.001$) were determined using one-way analysis of

variance (ANOVA) for an average of three to six replicates, followed by post hoc Tukey's test for all pair-wise mean comparisons.

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Author Contributions: S.M.M., A.G., and A.K. designed the study; S.M.M. synthesized the MA- κ -CA, produced the hydrogels, and performed the cells studies, swelling and degradation studies. S.M.M. and A.G. performed the rest of the experimental part; S.M.M., A.G. and A.K. wrote the paper. A.P.M., R.L.R., and M.E.G. revised the paper. All authors discussed the results and commented on the manuscript.

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