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Physicochemical Effects of PEG Content in Alginate-based Double Network Hydrogels as Hybrid Scaffolds

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Abstract: This study aims to prepare a double-network hydrogel as hybrid networks bearing both natural and synthetic polymers to obtain scaffolds with increased swelling capacity and tunable mechanical and morphological properties. Physically cross-linked alginate hydrogel was reinforced with various ratios of Poly(ethylene glycol) (PEG) polymers which were chemically gellated via UV light exposure with a water soluble initiator. Physicochemical properties of the resulting hydrogels were systematically investigated via Fourier-transform infrared (FT-IR) spectroscopy for chemical composition and Scanning Electron Microscopy (SEM) for their morphological features like porosity. Furthermore, the effect of PEG amount in the final hydrogel (10, 20 and 40%) on swelling capacity was evaluated as well as the rheological properties. Prepared double-network hydrogels were demonstrated to be composed of both natural alginate polymer and synthetic PEG chains in FT-IR spectrum. Although 10%PEG containing hydrogel was not significantly different in terms of swelling capacity from the alginate hydrogel alone, increasing PEG amount seems to have improved the swelling ability. Comparative reological studies presented that introducing covalently cross-linked PEG network into alginate one increased crosspoint of storage and loss moduli almost 12 times more providing a stiffer scaffold. Increasing PEG content decreased the pore size on SEM images, indicating more crosslinking points in hydrogel structure.

Key words: Hybrid Hydrogels, Physical Cross-linking, UV Curing, Porous Scaffolds.

Hibrit İskeleler Olarak Aljinat Bazlı Çift Ağ Hidrojellerindeki PEG İçeriğinin Fizikokimyasal Etkileri

Öz: Bu çalışma, artan şişme kapasitesine ve ayarlanabilir mekanik ve morfolojik özelliklere sahip iskeleler elde etmek için hem doğal hem de sentetik polimerleri taşıyan hibrit ağlar olarak çift ağlı bir hidrojel hazırlamayı amaçlamaktadır. Fiziksel olarak çapraz bağlı aljinat hidrojel, suda çözünür bir başlatıcı ile UV ışığına maruz bırakılarak kimyasal olarak jelleştirilen çeşitli oranlarda Poli(etilen glikol) (PEG) polimerleri ile güçlendirildi. Elde edilen hidrojellerin fizikokimyasal özellikleri, kimyasal bileşimleri için Fourier dönüşümü kızılötesi spektroskopisi (FT-IR) ve gözeneklilik gibi morfolojik özellikleri açısından Taramalı Elektron Mikroskobu (SEM) aracılığıyla sistematik olarak araştırıldı. Ayrıca reolojik özelliklerin yanı sıra son hidrojeldeki PEG miktarının (%10, 20 ve 40) şişme kapasitesine etkisi de değerlendirildi. Hazırlanan çift ağ yapılı hidrojellerin, FT-IR spektrumunda hem doğal aljinat polimerinden hem de sentetik PEG zincirlerinden oluştuğu gösterilmiştir. Her ne kadar %10 PEG içeren hidrojel şişme kapasitesi açısından tek başına aljinat hidrojelden önemli ölçüde farklı olmasa da, artan PEG miktarının iyileştirici etkisi vardır. Karşılaştırmalı reolojik çalışmalar, kovalent olarak çapraz bağlı PEG ağının aljinata dahil edilmesinin, depolama çapraz noktasını ve kayıp modülünü neredeyse 12 kat artırdığını ve daha sert bir yapı iskelesi sağladığını ortaya koydu. PEG içeriğinin arttırılması, SEM görüntülerinde gözenek boyutunun azalmasına neden oldu ve bu da hidrojel yapısında daha fazla çapraz bağlanma noktasının olduğunu gösterdi.

Anahtar kelimeler: Hibrit Hidrojeller, Fiziksel Çapraz Bağlama, UV Kürleme, Gözenekli İskeleler

1. Introduction

Regarding the rapidly growing field of tissue engineering, the need for innovative biomaterials remains very significant and is desired to address the complexities of mimicking native tissue microenvironments [1,2]. As powerful scaffold platforms, hydrogels have emerged as very advantageous scaffolds for three-dimensional (3D) networks, owing to their inherent biocompatibility, tunable mechanical properties and porosity levels, together with their potential for controlled release of biomolecules like bioactive agents and growth factor [3,6]. Although there are numerous hydrogel designs in the literature, most of them depend on either physical interactions like electrostatic interactions or self-assembly of amphiphilic polymers or chemical cross-linking like UV-based radicalic gelation or wellknown coupling reactions such as NHS/EDC coupling dependent amidation, 1,4 Michael addition or click reactions [7,14]. With the need for better control on hydrogel scaffolds by improving the complexity coming from the composite designs, it has been recently switched to combine both natural and

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synthetic polymeric biomaterials with different cross-linking strategies to come up with more advantageous polymeric networks.

Double network hydrogels are such systems providing step-wise cross-linking ability of different types of materials in a more controlled fashion [15,16]. Each part can be cured independently from each other and this orthogonal cross-linking strategy can be seen as a new, yet more powerful tool to obtain more tough and biomimetic scaffolds for the target tissue modeling [17,19]. In the literature, there are several examples of interpenetrating networks that involve two different types of polymers, one of which is cured and the other one is entrapped in the generated pores [20,21]. However, based on their degradation tendencies and pore size, the mechanical properties of the resultant scaffold might be weaker than desired. The above-mentioned hydrogel systems are obtained by orthogonally cross-linked two polymeric biomaterials in the same environment in a sequential or simultaneous fashion so that the resultant scaffold becomes more stable and modular, enabling more possibilities for the adjustment of final properties of the obtained scaffold [22].

Literature reveals that the double-network hydrogels have been mostly prepared by the combination of natural polymers. Alginate, a naturally occurring polysaccharide derived from brown seaweed, possesses inherent biocompatibility and gel-forming properties based on electrostatic interactions with divalent cations like Ca^{+2}, making it an attractive candidate for tissue engineering applications [23]. Despite their biomimetic features for a solid tissue, high degradation rate and low stability require the urgent need for the contribution of synthetic, yet biocompatible polymers, among which biodegradable polyesters have a very limited use due to their hydrophobicity. On the other side, PEG (Poly(ethylene glycol)) remarkably stands as a potential solution for both introducing the increased hydrophilicity to the final scaffold structure and providing more options for cross-linking process with its versatile functional groups. In 2015, Chee et al. presented the preparation of alginate hydrogel integrated with polymerized PEG monomethyl methacrylate, which was cured under UV light to obtain injectable gel-like material [24]. However, the PEG chains in this study were appended to the main polymer backbone, hence less likely to contribute to the stiffness of the final scaffold. In a very recent study released in 2023, Zhu and coworkers prepared alginate/PEG double network hydrogels containing anthracene-functionalized 4-arm PEG polymer which crosslinks based on the UV-mediated dimerization of terminal anthracene moieties [25]. This structure was utilized for obtaining patterning of resultant hydrogel with photolithography and may not have a great potential for bio-applications due to the toxicity of anthracene groups [26]. As a more relevant study, Huang et al. has shown the alginate/PEG based double network hydrogels, prepared by different weight ratios of alginate in PEG-based scaffold [16]. Although they have concluded that the contribution of alginate to the final hydrogel structure helped to get smaller pores and improve mechanosensing against adipose derived stem cells, taking PEG network as the basis and introducing alginate polymer with a relatively higher molecular weight would complicate the reproducibility of the final construct as PEG length and branching degree increases. As a more relevant study, Hong et al. prepared hydrogels with interpenetration of only 40% acrylated PEG chains in alginate network, supporting the idea of this polymer pair to obtain highly stretchable and though scaffolds [27].

Based on the lights of these findings, the study presented in this paper suggests a systematic approach for the preparation of alginate/PEG double network hydrogels, which takes the alginate hydrogel as the main network and reinforce it with the UV-light mediated crosslinking of dimethacrylated linear PEG chains of various ratios in alginate network depending on the ionic interaction with the added $CaCl_2$ solution. By incorporating PEG, a synthetic polymer known for its versatility and hydrophilicity, into the alginate network, this demonstrated double-network hydrogels provide better control over the synthetic part of this hybrid scaffold with tunable features for swelling capacity, mechanical strength and porosity (Fig. 1). The prepared double-network hydrogels containing a natural polymer, alginate, and a synthetic one, PEG, aimed to benefit from their synergistic advantages to develop more stable and biomimetic environments for further tissue engineering applications. Characterization results for the obtained hydrogels with varying PEG content revealed that increased PEG amounts have enhanced the swelling ability of scaffolds tremendously and improved their mechanical properties with micron-sized pores visualized under SEM.

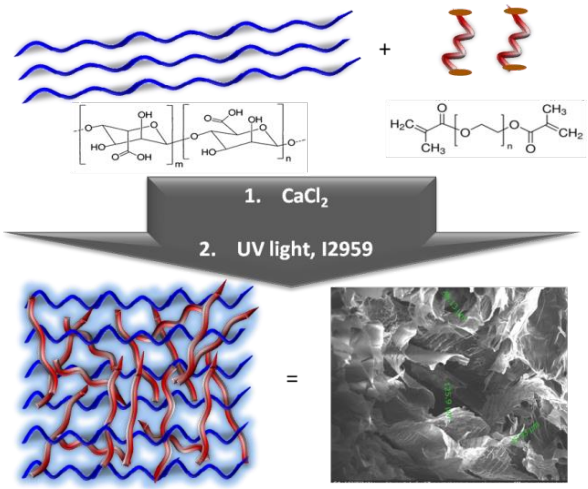

Figure 1. General scheme for the preparation of Alginate/PEG double-network hydrogel.

2. Materials and Methods

Materials and Instrumentation

Alginic acid sodium salt from brown algae-medium viscosity was purchased from Sigma-Aldrich. Poly(ethyleneglycol) dimethacrylate (PEGdiMA) was obtained from Sigma-Aldrich, with an average Mn=750 $gmol^{-1}$and contains 900-1100 ppm MEHQ as inhibitor, which was removed by passing through basic alumina column prior to use. Calcium Chloride ($CaCl_2$) and Irgacure 2959 (98% purity) were purchased from Merck Millipore. All used organic solvents were reagent-grade.

FT-IR spectroscopy measurements were done on a Perkin Elmer Spectrum Two FT-IR spectrometer. For SEM visualization, conductive carbon tabs and mounts were obtained from Ted Pella, and samples were prepared as coated with 20 nm of gold (Au/Pb) under vacuum. Images were taken under low vacuum at Thermo Fisher Scientific (FEI) Quattro S ESEM. Mechanical evaluation of hydrogels was performed by Malvern Kinexus Pro and J2 SR 4703 SS geometry, obtained from ©Malvern, was used in rheological experiments. The strain-dependent oscillatory rheology measurements of hydrogel scaffolds (500 mg for each sample)was tested at f=1Hz and with γ=0.01, at 37 ºC (30 data points).

Preparation of Alginate/PEG Double-Network Hydrogel (AA/PEG-HG)

Only alginate polymer containing control hydrogels (AA-HG) were prepared by physical cross-linking of alginate chains with Ca^{+2} ions in aqueous environment. Briefly, alginate polymer (100 mg) was dissolved in ddH_2O (10mL) to obtain a solution of 1% by weight. On the other side, 75 mM $CaCl_2$ solution was prepared in ddH_2O, as well [28]. Their equi-volume mixture was prepared to obtain a homogeneous solution and then let to shake at 500 rpm in an orbital shaker for an additional 10 minutes for complete gelation. Obtained hydrogels were washed with fresh ddH_2O for three times and obtained transparent hydrogel structures were freeze-dried in lyophilizer for further characterization steps.

Double network hydrogels were prepared by the incorporation of PEGdiMA polymers into the hydrogel environment with different ratios by volume. Increasing amounts (10, 20 and 40%) of PEGdiMA polymer, determined with respect to the final solution volume, were mixed with the alginate solution. After the addition of 1% Irgacure (by weight, with respect to PEGdiMA), the final solutions were mixed with $CaCl_2$ solution by equal volume homogeneously and as stated for control hydrogel above, complete gelation of alginate part was obtained after its incubation for 10 minutes in the orbital shaker. Later on, these mixtures were directly exposed to UV light (365 nm) for 20 minutes for the chemical cross-linking of PEGdiMA polymers [28,29]. Then, obtained doublenetwork hydrogels were washed with fresh ddH_2O for three times and obtained scaffold were freeze-dried in lyophilizer for further characterization steps.

3. Results and Discussion

The preparation of alginate/PEG based double network hydrogels were achieved by physical cross-linking of negatively charged alginate chains with a divalent cation Ca^{+2} in aqueous environment, followed by UV light mediated chemical cross-linking of PEG polymers functionalized with methacrylate moieties at its both ends. Different ratios of PEGdiMA polymers were added into the alginate solutions and after step-wise crosslinking procedures, the obtained double-network hydrogel constructs were investigated for the effect of immobilized PEG chains interconnected in an alginate mesh on gel conversion, swelling capacity, mechanical properties and porosity. The adjustability of PEG amount in the prepared double-network scaffolds clearly provide a modular approach for tuning the final mechanical, morphological and swelling properties of alginate-based hydrogels while still preserving its major biomimetic features.

Step-wise gelation procedures were followed by slight modifications from the literature for the mixture of alginate and PEGdiMA polymers with $CaCl_2$ and Irgacure, respectively. The obtained hydrogels were weighed for their final amounts to calculate gel conversion, based on the equation 1. Obviously, negatively charged carboxylic acid groups of alginate polymers make electrostatic interactions with the Ca^{+2} ions included into the media readily, to give a higher yield for the gelation of alginate chains (Table 1). However, the addition of PEGdiMA polymer seems to decrease the total gel conversion, which might be due to the slower radical generation by the water soluble photoinitiator, Irgacure. Also, taking the relatively smaller molecular weight into account, it can be estimated that some of the PEGdiMA chains were not included into the gelation, when gel conversions of double network hydrogels were compared to the control hydrogel (Eq. (1)).

$$\text{Gel Conversion} = \left[\frac{(\text{Weight of obtained hydrogel})}{(\text{Weight of polymers used})}\right] * 100\text{Gel Conversion} = \text{Weight} \quad (1)$$

Table 1. Preparation details and calculated gel conversion for the obtained hydrogels.

No	Code	V of 1% Alginate (mL)	% of PEGdiMA (v/v)	Amount of PEGdiMA (µL)	V of 75 mM $CaCl_2$ (mL)	Gel Conversion
1	AA	0.5	0	0	0.5	96
2	AA-10PEG	0.5	10	50	0.5	81
3	AA-20PEG	0.5	20	100	0.5	71
4	AA-40PEG	0.5	40	200	0.5	82

Although alginate polymer is very hydrophilic and readily dissolves in water, the hydrogel dependent on its physical cross-linking demonstrate a low stability for the final construct. This might be due to not only the high degradation rate of alginate polymers in aqueous media but also their dissociation upon the removal of Ca^{+2} ion from the gel part, based on their diffusion to outside environment. Addition of chemically cross-linked PEG chains into environment, the resultant hydrogel structures were expected to preserve more stability in water, with an increased swelling capacity and lower degradation profile. With this aim, prepared AA/PEG double network hydrogels were incubated in pH7.4 PBS (phosphate buffer solution) and at body temperature (37 ºC) for a certain period of time to mimic the physiological conditions. At different time points, the hydrogels were removed from the solution and excess water was removed. The weight of hydrogels was recorded and the swelling ratio for the hydrogels was calculated by the Eq. (2).

$$\text{The swelling ratio} = \left[\frac{\text{Weight of wet hydrogel} - \text{Weight of dry hydrogel}}{\text{Weight of dry hydrogel}}\right] * 100 \quad (2)$$

Fig. 2 demonstrates that addition of PEG chains into the final hydrogel structure leads to an increase in the swelling ratio value. Compared to only alginate hydrogel, the capacity of hydrogels for water uptake capacity has improved with the increased amount of PEG chains incorporated into the hydrogel. 10% PEG containing hydrogel seems to show no significant difference for swelling capacity, however for 20 and 40% PEG containing ones, it is clearly seen that the swelling capacity of the resultant double-network hydrogels was dramatically increased. After 3-4 hours, the weight of hydrogels were observed to decrease, which might be due to its

degradation, and it is notable that the point at which the decrease started was later for PEG containing double-network hydrogels, compared to only alginate one. This might be an indication for the ameliorating effect of chemically cross-linked PEG network on the stability of the final scaffold structure.

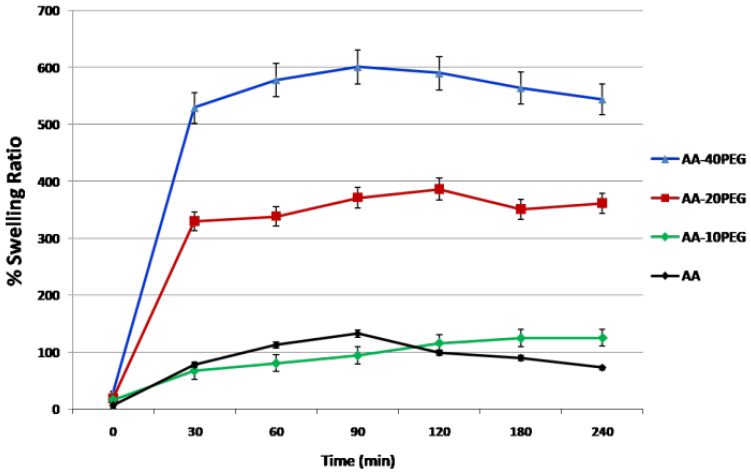

Figure 2. Swelling ratios of prepared hydrogels.

For the chemical structure analysis of the prepared hydrogels, FT-IR spectroscopy was used to compare the characteristic peak evaluations of the polymers separately and they are compared with the peaks observed at the spectra of double-network hydrogels. Fig. 3 clearly reveals that the spectra for AA-20PEG hydrogel demonstrates both the peaks belonging to alginate polymer as singlet at 1595 cm^{-1} for C=O stretching for carboxylic acid group and a broad multiplet between1020-1070 cm^{-1} for C-O bonds. The broad peak seen at around 3300 cm^{-1} represents the presence of labile proton based bonds in hydrogel structure, coming from the alginate polymer. On the other site, the presence of a sharp peak at 1736 cm^{-1} comes from the ester carbonyl double bond in methacrylated ends, verifying the incorporation of PEG chains into the hydrogel structure. Moreover, the disappearance of peak for hydrogel spectrum, which appears at 1638 cm^{-1} in PEGdiMA one, points to the removal of C=C methacrylate double bond upon radicalic UV cross-linking. This chemical structure evaluation for double-network hydrogel indicates the successful network formation by PEGdiMA chains in alginate network.

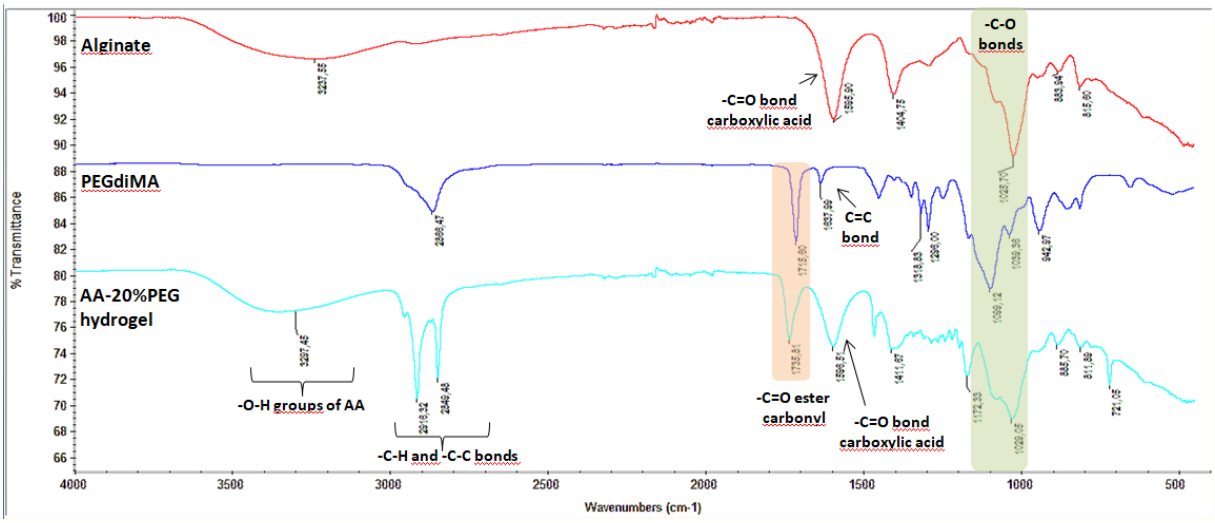

Figure 3. FT-IR spectra of Alginate polymer, pure PEGdiMA polymer and 20%PEG containing alginate (AA-20PEG) hydrogel.

The ionic interaction based physical cross-linking of alginate polymer chains are expected to provide a porous structure, which was visualized by SEM. Fig. 4 clearly shows that alginate hydrogels provide a homogenously distributed pores with mostly 150-200 µm in diameter. Incorporation of chemically cross-linked PEG chains obviously decreases the pore size of the resultant double-network hydrogel, indicating to the increased cross-linking degree in the scaffold. It can be noted that the greater the amount of PEGdiMA chains wasin the hydrogel, the smaller the generated pores formed to appear around 50 µm. Especially the SEM image for AA-40PEG hydrogel provides a very clear look to very regular pore distribution, due to the high amount of PEG chains homogeneously mixed in alginate network, indicated with red arrows. These images confirm the efficient crosslinking of PEG chains in alginate network, which can be used as a powerful tool to modulate its morphological properties like porosity and pore size.

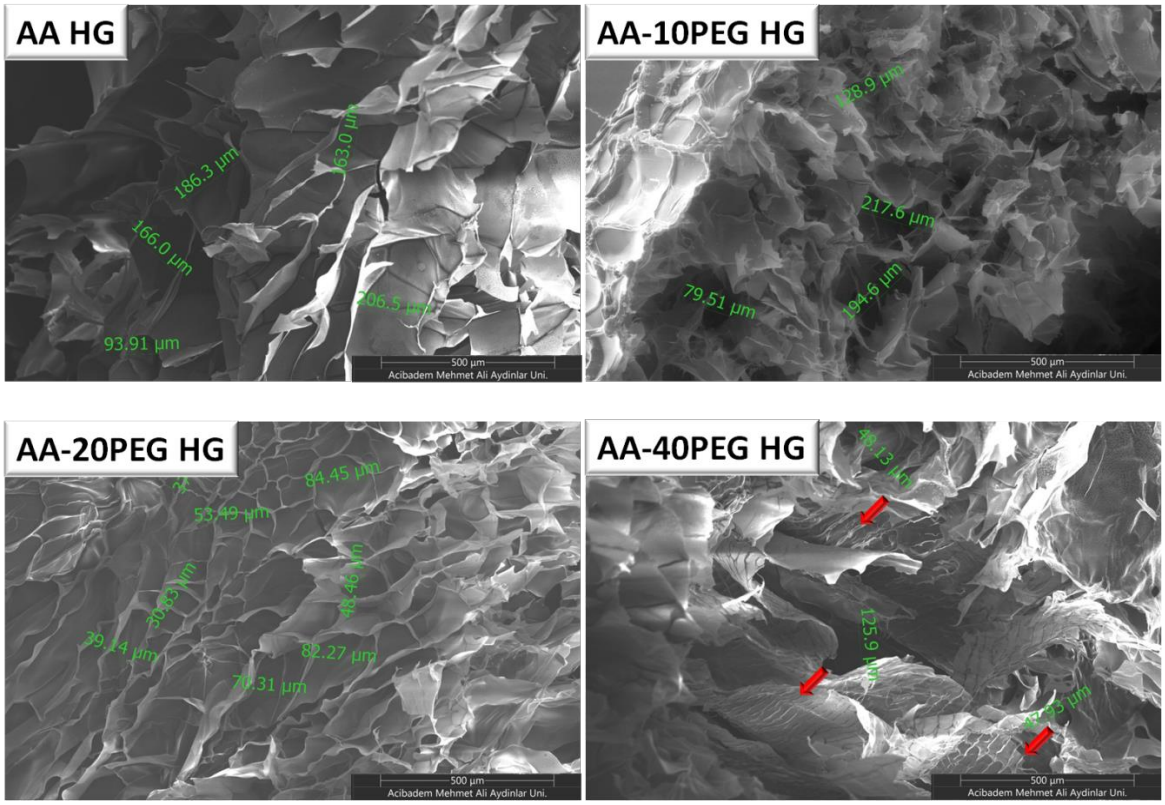

Figure 4. SEM images for prepared double-network hydrogels and their comparison with only alginate hydrogel.

One of the most commonly encountered issues for soft-tissue resembling hydrogel scaffolds is their weak mechanical properties, which have a determining impact on further cell attachment in tissue-engineering applications. Reinforcing these properties with increasing the stability of the final construct could be achieved by the incorporation of comparably less degrading synthetic polymers into the network, which was the strategy applied in this study. As a natural and biocompatible polysaccharide, alginate polymer provides a very good biomimicry for soft tissues, from which cannot be benefitted with a high yield because of its less stability and fast degradation profile in aqueous environment. In this work, we aimed to improve the mechanical stability of alginate polymer to create scaffolds with tunable stiffness by adding desired amount of PEG chains in hydrogel structure, without giving up on the main properties of alginate network. This trend of prepared double-network hydrogels were analyzed by a rheometer and compared with that of control alginate hydrogel. Fig. 5 provides the trends for both G' (storage modules) and G'' (loss modulus) for the hydrogels, where the former one represents the elasticity (solid-like structure) of the scaffolds and the latter one is for the viscosity (liquid-like structure). The strain-dependent oscillatory rheology measurements of hydrogel scaffolds was tested at f=1Hz and with γ=0.01, at 37 ºC. Compared to the alginate hydrogel alone, PEG containing double-network hydrogels provide an increased G' storage modulus value, indicating enhanced stiffness of the final constructs, which gets better with an increased amount of PEG (especially for 20 and 40 % content). Also, the cross point values (G'/G'') are

seen to be shifted to right, almost 12 times more compared to only alginate hydrogel, strengthening the idea of getting stiffer and more elastic for the obtained double-network hydrogels with better mechanical properties. It is more clear for 40%PEG containing alginate hydrogel, G' curve lasts up to γ=100 which indicates the hydrogel gets more viscous (liquid-like) structure at a higher strain value and withstand with more deformation compared to other hydrogels.

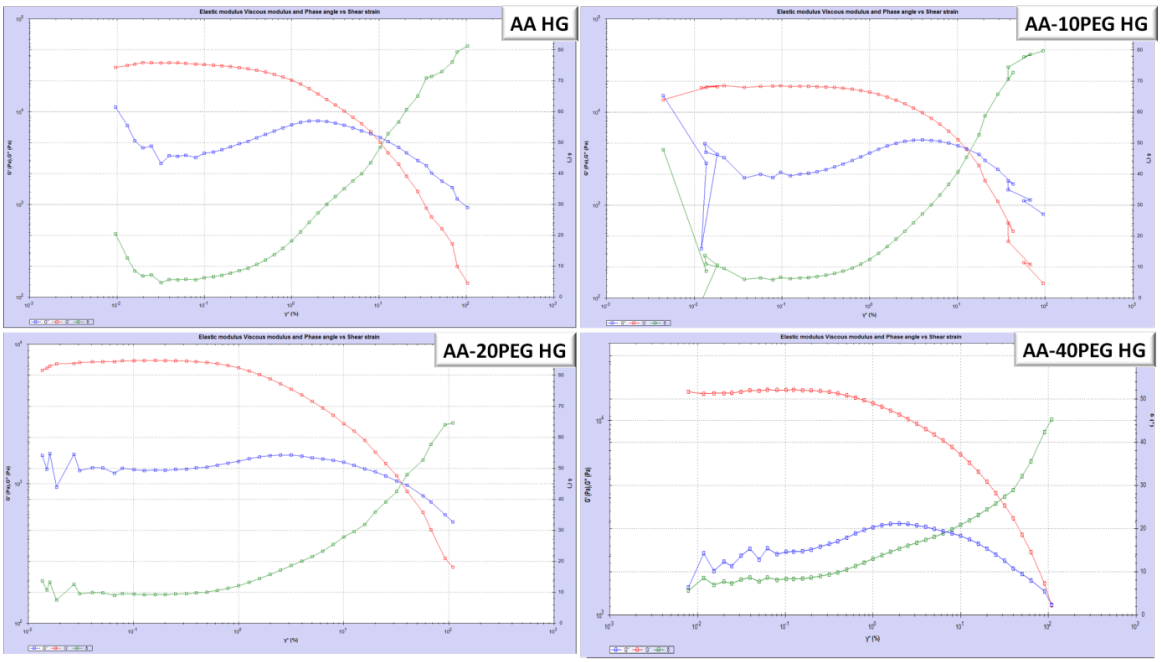

Figure 5. Mechanical evaluations for prepared double-network hydrogels and their comparison with control alginate hydrogel. (Red curve represents G' (storage modulus), Blue curve represents G'' (loss modulus))

4. Conclusion

In summary, double-network hydrogel platforms were prepared with high yield (>70%) by physical cross-linking of alginate polymers with Ca^{+2} ions, followed by chemical cross-linking of PEGdiMA chains under UV light. Alginate-based hydrogels were aimed to have better properties in terms of their swelling ability, and mechanical and morphological features without losing their biomimicry by the addition of various amounts of PEG polymers (10, 20, and 40% by volume), which were immobilized in alginate network to demonstrate the effect of incorporation of a hydrophilic and synthetic component into the scaffold structure. While these double-network hydrogels were characterized for their hybrid chemical composition successfully with the expected peak in FT-IR spectra, their improved mechanical properties as increased G' value and swelling capacity up to 600% clearly indicate the benefit of PEG content. Morphological evaluations also verify that the porosity was directly related to the increased PEG amount and its cross-linking density, which was clearly seen for 40%PEG containing hydrogels, compared to only alginate hydrogel. Overall, these hybrid scaffolds were obtained as reinforced double cross-linked networks with tunable properties by adjusting the PEG amount inside the alginate polymer, which provides a high potential to prepare the optimum scaffolds for more biomimetic environments for further tissue-engineering applications.

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