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Studies on Novel Methods for Formulating Novel Cross-Linked Hydrogel Films of Hyaluronic Acid

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Abstract: Hyaluronic acid (HA) is a natural polysaccharide with promising applications in modern cosmetic and nutricosmetic products due to its high-water affinity, which is essential for skin hydration, as well as its biocompatibility, biodegradability, non-toxicity, and non-immunogenic nature. In this study, we investigated and optimized the method of crosslinking for formulating novel HA hydrogel films. We used Pentaerythritol Tetra-acrylate (PT) as the cross-linking agent over a range of pH values and used different cross-linking methods (Ultraviolet (UV) radiation, microwaving, and oven heating). The efficacy of the cross-linking reaction was evaluated using swelling studies and Fourier transform infrared (FTIR) spectroscopy for the characterization of the xerogel HA-PT film formulations. We found that HA-PT cross-linked hydrogels are produced under alkaline conditions (pH 11) but not under neutral or acidic conditions. Cross-linked HA-PT xerogel films using UV-irradiation showed excessive swelling indicative of inadequate cross-linking. The oven and microwaving methods produced HA-PT films with high cross-linking density. FTIR data suggest formation of ester bond between the carbonyl of the HA and hydroxyl group of the PT acrylate group. Overall, the oven method was considered better and easier than UV-radiation/microwave methods because it is safer, user-friendly and eco-friendly, and can process larger batches.

Keywords: Hyaluronic acid; Pentaerythritol Tetraacrylate; cross-linker; hydrogels; UV radiation; microwave; oven; FTIR; swelling; cosmetics

1. Introduction

Cross-linked hydrogels are three-dimensional (3D) networks of polymer chains, containing hydrophilic groups or domains that can uptake and retain significant amounts of water [1]. The cross-linking bond can be classified as chemical (Type 1 hydrogels) or physical (Type 2 hydrogels). Chemical cross-linking involves stable covalent bonds and is, therefore, irreversible. By contrast, physical cross-linking involves a network of molecular entanglements, and/or secondary forces including ionic, hydrogen bonding, or hydrophobic interactions. These interactions can be reversed/broken by changes in physical conditions or on application of stress [1]. Therefore, chemical crosslinking enhances the mechanical stability of hydrogels, which leads to hydrogel film formulations [2]. Water uptake and water holding capacity are the most important characteristics of cross-linked Type 1 hydrogels.

Natural biopolymers such as alginate, chitosan, hyaluronic acid (HA), collagen, and gelatin have been used to formulate a variety of hydrogels for cosmetic and pharmaceutical applications [3]. Hyaluronan or Hyaluronic acid (HA) is a natural linear polymer with promising biomedical and cosmetic applications. It contains repeated units of a disaccharide of β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine (Figure 1a). It occurs naturally in vivo as a polyanion in vitreous body fluids of the vitreous humour of the eye, synovial fluid of articular joints, and connective tissue [2,4,5].

It functions to space-fill, lubricate, and regulate the hydro-physiologic environment in which many cell activities occur including cell growth, migration, and differentiation [2,4,6]. It is a highly biocompatible, biodegradable, nontoxic, and non-immunogenic polymer with high water affinity and, therefore, an attractive biomaterial. HA exhibits tissue-reparative effects in a normal wound-healing process by promoting cell migration, proliferation, and infiltration of leukocytes as well as tissue hydration [7]. It is extensively used in ophthalmic surgery, osteoarthritis injection treatment, dermal fillers for tissue augmentation, and cutaneous wound healing [2,6,7]. However, the reparative effects of HA depend, in part, on its molecular weight (MW). Low MW HA (1300–6800 g/mol) grades are effective in stimulating proliferation of endothelial cells and immunologic responses, whereas high MW HA can inhibit the formation of new blood vessels (angiogenesis) [7].

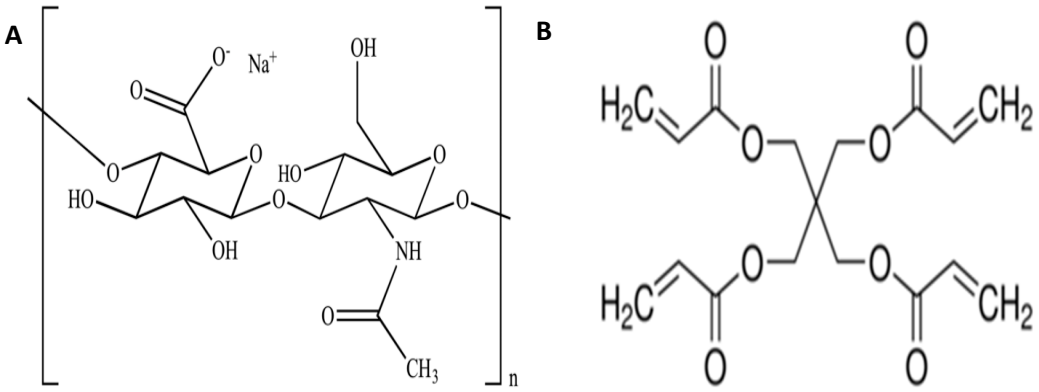

Figure 1. Chemical structures of: (**A**) sodium Hyaluronate and (**B**) pentaerythritol tetra-acrylate.

HA is increasingly becoming a key ingredient in modern cosmetic and nutricosmetic products due to its skin rejuvenating effects [8]. HA-based cosmetic formulations including gels, autologous fat gels, dermal/intra-dermal filler injections, creams, lotion, and serum have been shown to exhibit noticeable space-filling, anti-wrinkle, anti-aging, anti-nasolabial fold, and, overall, face rejuvenating properties [8]. However, the maximum range of potential cosmetic applications for HA is yet to be achieved due to some of its undesirable chemical properties. For instance, HA exhibits suboptimal efficacy due to its inability to form physical (Type 2) hydrogels over a wide range of pH conditions. Additionally, it has a short half-life of about 12 h as it undergoes rapid degradation by the hyaluronase enzymes present in body tissues [5,6]. Therefore, cross-linked (Type 1) HA hydrogel films can overcome the above limitations and provide a robust cosmetic platform. They have lower enzymatic degradation rates and lower swelling ratios compared to linear polymers due to the presence of covalent bridges and intermolecular bonds/forces between the polymer chains and the chemical cross-linker.

The hydroxyl (-OH), carboxylic (-COOH), and amide (-$NHCOCH_3$) functional groups in HA are available for crosslinking via an ether bond (R-O-R), ester linkage (R-COO-R), and carbodiimide, respectively. Therefore, HA has been successfully cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), glutaraldehyde (GTA), poly (ethylene glycol) diglycidil ether (PEGDE), ethylene glycol diglycidil ether (EGDE), and divinyl sulfonate (DVS) among others as crosslinkers [6]. To maintain the biocompatibility of cross-linked HA, the effective proportion of a cross-linker should be as low as possible. Pentaerythritol tetra-acrylate (PT) (Figure 1b), which is used as a binding agent, a solvent, a colorant, and a fragrance for a variety of pharmaceutical and cosmetic applications, is a promising crosslinking agent. It has been used to formulate alginate-based hydrogels [9]. PT is a highly photosensitive chemical than can readily undergo photo-polymerisation with radicalized molecules. It has recently been used to crosslink polyethylene oxide (PEO) hydrogel films synthesised via UV-radiation [10]. In our work, apart from UV, we also trialled microwave irradiation and oven-assistant thermal crosslinking using Pentaerythritol Tetra-acrylate PT as the crosslinking agent with HA [11].

There are several quality parameters for hydrogels, which include gel fraction (the insoluble part of the dried sample following hydration in de-ionised water for 16 h) and gel swelling. For instance, an increase in PT proportions in PEO-PT hydrogels were shown to significantly increase the gel fraction and crosslinking density, while causing a significant decrease in the equilibrium water capacity, average MW between cross-links, and mesh size [10]. Key analytical assays for the characterisation of cross-linked hydrogels include swelling ratio and mesh size. In addition, cross-linked polymers are commonly analysed by Fourier Transform Infrared Spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR) [1,5]. FTIR and NMR are useful for identifying chemical changes, structural arrangements, and possible cross-linking mechanisms in hydrogels by comparing the chemical structure of the gel with those of its constituents [1].

The aim of the present work was to explore the synthesis of novel cross-linked HA-PT hydrogel films using PT as the cross-linking agent and to optimise the formulation and reaction process by evaluating the effects of the following independent variables on cross-linking.

- pH of the formulation mixture before cross-linking,
- PT concentration (10%, 15%, 20%, and 30% w/w) in the formulation,
- type of cross-linking method (UV-irradiation, microwaving, and oven-assisted crosslinking),
- exposure time of the film to the cross-linking method.

The efficacy of the cross-linking reaction was evaluated using swelling studies (dependent parameters) and Fourier transform infrared (FTIR) spectroscopy for the characterization of the xerogel HA-PT film formulations.

In contrast to other reported methods [6], our approach is intended to avoid additional functionalisation of the HA prior to or during the cross-linking reaction, which avoids the use of organic solvents and multiple reaction steps. As such, our overall aim is to provide a simple and robust one-step crosslinking method for high MW HA.

2. Materials and Methods

2.1. Materials, Chemicals, and Reagents

Hyaluronic acid (HA) sodium salt with high molecular weight (1800–2200 KDa) was supplied by Infinity Ingredients (Binfield, UK), while PT was purchased from Insight Biotechnology Limited (Middlesex, UK). These materials were used as received unless otherwise described. Other chemicals and reagents included NaOH (1.0 M) and HCl (1.0 M), which were used for pH adjustment. Deionized distilled water was available in the laboratory and was used as solvent for the HA polymer and as a polar swelling agent for the HA-crosslinked films.

2.2. Preparation of Hyaluronic Acid Hydrogels

HA-based hydrogels were formulated with different concentrations of HA and PT, as summarized in Tables 1 and 2. The hydrogels were prepared by dissolving HA in deionized distilled water (neutral pH 7, Table 1), or HCl-adjusted water (acidic pH 2, Table 1) or NaOH-adjusted water (alkaline pH 11, Table 2). The mixtures were stirred with an IKA stirrer (IKA® Werke GmbH. & Co. KG, Staufen, Germany) for 6 h to obtain homogeneously mixed HA hydrogels. This was followed by adding different amounts of PT, where the mixture was subsequently stirred slowly for 24 h to obtain completely homogenized HA-PT hydrogels. The hydrogel was left to stand for another 24 h to release air bubbles before casting in Petri dishes. The cast hydrogel samples were air-dried at room temperature for four to five days. The obtained xerogel films were cut in small pieces for the cross-linking experiment. The nomenclature of each xerogel indicates the concentration of HA in the initial aqueous solution and the concentration of PT in the xerogel, e.g., HA2-PT20 means that HA was dissolved in a concentration of 2% w/v in the initial aqueous mixture and that the concentration of PT in the final product (xerogel) was 20% w/w (Tables 1 and 2).

Table 1. Hydrogels formulated with HA and PT in neutral and acidic pH conditions.

Hydrogel Name	% w/v of HA in Aq Solution	% w/w of PT in HA	pH Condition	PT: HA Ratio in the Xerogel
HA2 *	2	——	7	—-
HA2-PT5 *	2	5	7	1:20
HA5-PT5 *	5	5	7	1:20
HA2-PT10 *	2	10	7	1:10
HA5-PT15 *	5	15	7	1:6.7
HA5-PT20 *	5	20	7	1:5
HA2 *	2	——	2	—-
HA2-PT5 *	2	5	2	1:20
HA5-PT5 *	5	5	2	1:20
HA2-PT10 *	2	10	2	1:10
HA5-PT15 *	5	15	2	1:6.7
HA5-PT20 *	5	20	2	1:5

* film dissolved during the swelling test.

Table 2. Hydrogels formulated with HA and PT in alkaline pH conditions (pH = 11).

Hydrogel Name	% w/v of HA in Sq Solution	% w/w of PT in HA	PT: HA Ratio in the Xerogel
HA2 *	2	—	—
HA2-PT5 *	2	5	1:20
HA5-PT5 *	5	5	1:20
HA5-PT10	5	10	1:10
HA5-PT15	5	15	1:6.7
HA5-PT20	5	20	1:5
HA5-PT30	5	30	1:3.3

* film dissolved during the swelling test.

2.3. Cross-Linking Experiment

Cross-linking of the HA-PT xerogel films was performed by three different techniques: (1) UV-light irradiation, (2) microwave-irradiation, and (3) oven-assisted thermal crosslinking.

2.3.1. UV-Light Irradiation

Hydrogel cross-linking by UV-light irradiation was performed using 150 W medium pressure mercury lamp (TQ 150, Heraeus Noble light GmbH, Hanau, Germany). The UV emission spectrum was in the range of 248–579 nm, with a maximum wavelength (λ max) of 366 nm. Each of the xerogel pieces was individually placed in the inner glass wall of the UV reactor, where they were irradiated at different intervals (10, 20, 30, and 40 min). The films were irradiated on both sides.

2.3.2. Microwave Irradiation

In this experiment, individual pieces of HA-PT xerogel films were placed in the middle of the rotating glass plate in the chamber of the microwave (Panasonic NN-E281MM, Panasonic UK Ltd., UK). The microwave irradiation output power used was low (800 watts). The degree of cross-linking of the films was evaluated at different time intervals, which were 10, 20, 30, 40, and 60 min.

2.3.3. Thermal Cross-Linking

Oven-assisted thermal cross-linking of the HA-PT xerogel films was performed using an 80 °C oven (Binder GmbH Berg ster, 14 D-78532 Tuttlingen). The xerogel film samples were placed in the middle of the oven shelf for 24 h.

2.4. Hydrogel Swelling Studies

Three samples (n = 3) of the cross-linked xerogel films from each batch (different cross-linking technique) were accurately weighed (*Mo*) and then were swollen in distilled water for 24 h at room temperature. During swelling, the films were periodically removed from water, excess surface water

was drip-dried, and the films were reweighed (*Ms*). The percentage swelling (%) was calculated using Equation (1) [10] and the maximum swelling was calculated using Equation (2) [10]. After 1 h, they reached equilibrium.

$$\% \, Swelling = \frac{Ms - Mo}{Mo} \times 100 \tag{1}$$

$$\% \, EWC = \frac{Ms - Mo}{Ms} \times 100 \tag{2}$$

where *Mo* was the initial weight and *Ms* was the weight of the swollen film at equilibrium.

The thickness of each swollen cross-linked hydrogel film was measured using a micrometer screw gauge (Duratool, Taichung, Taiwan). The gel fraction for each film was calculated using Equation (3).

$$Gel \, Fraction \, (\%) = \frac{M'}{Mo} \times 100 \tag{3}$$

where *M'* is the weight of film after drip-drying excess water.

2.5. Comparisons of Average MW between Crosslinks, Crosslinking Density, and Mesh Size

The average MW between cross-links ($\overline{M}_c$), cross-linking density (*Ve*), and mesh size (ξ) of the hydrogel formulations were calculated according to the equilibrium swelling theory, which assumes Gaussian distribution of cross-linked polymer chains. The Flory Rehner Equation (4) was used to calculate $\overline{M}_c$ [12].

$$Q_v^{5/3} = \frac{\overline{v}\overline{M}_c}{v_1}\left(\frac{1}{2} - x\right) \tag{4}$$

where $Q_v^{\frac{5}{3}}$ is the volumetric swelling ratio, $\overline{v}$ is the specific volume of the dry polymer ($\overline{v}$ = 0.575 cm^3/g for HA), v_1 is the molar volume of the solvent (18 cm^3/mol for water), and x is the Flory polymer solvent interaction parameter (for HA). The x value was estimated to be 0.473 based on several assumptions [12,13]. Qv was calculated as Q_m, which is the degree of mass swelling, according to Equation (5).

$$Qv = 1 + \frac{Pp}{Ps}\left(Q_m - 1\right) \tag{5}$$

where, *Pp* is the dry polymer density (1.229 g/cm^3) and *Ps* is water density. Q_m is the swelling ratio using the weights of the film before (xerogel) and after swelling. Q_m was used to calculate Qv.

Equations (6) and (7) were used to calculate the effective crosslinking density (*Ve*) and swollen hydrogel mesh size (ξ), respectively.

$$Ve = \frac{Pp}{\overline{M}_c} \tag{6}$$

$$\xi = Qv \sqrt[\frac{1}{3}]{ro2} \tag{7}$$

where $\sqrt{ro2}$ is the root-mean square (RMS) distance between crosslinks, which is dependent on the MW between crosslinks. For HA, a RMS distance value was used as previously calculated (Equation (8)) [12].

$$\left(\frac{ro2}{2n}\right)^{1/2} \cong 2.4 \text{ nm} \tag{8}$$

where n is the polymeric disaccharide units for HA with a given MW. For HA with an n of 5305 and MW of 2×10^6 g/mol, RMS was calculated as follows (Equation (9)).

$$\sqrt{ro2} = 0.1748 \sqrt{\overline{M}_c} \, Qv^{1/3} \text{ (nm)} \tag{9}$$

Lastly, combining Equations (7) and (9) and substituting $\overline{M}_c$ for *Mn* gives Equation (10).

$$\xi = 0.1748 \sqrt{\overline{M}_c}\ Qv^{1/3}\ (\text{nm}) \quad (10)$$

2.6. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR was done at room temperature using Shimadzu IR Affinity-1S Fourier Transform Infrared Spectrometer (Shimadzu UK Ltd., Milton Keynes, UK) for all film batches to evaluate the cross-linking degree of HA-PT xerogel films. FT-IR analysis was also carried for pure HA and pure PT. The spectral range was 4000–550 cm^{-1} with a resolution of two wave numbers (cm^{-1}).

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism V. 8.0 (GraphPad Software, Inc., San Diego, CA, USA). The resulting percent swelling and mesh size data of the hydrogel samples and treatments were compared using One-Way Analysis of Variance (ANOVA) with Tukey's multiple-comparison post-test. Paired t-test was used to compare treatment effect within samples. Differences within and between treatments were considered to be significant at a p value of <0.05.

3. Results and Discussions

3.1. Hydrogel Characterization

Figure 2 below shows the appearance of the HA5-PT20 films before and after swelling, which provides visual evidence of the hydration of the film. The swelling occurred in all dimensions including length, width, and thickness of the film, which was also demonstrated for 5HA-5GAN (GAN, Gantrez® S-97) by Larraneta et al. [2].

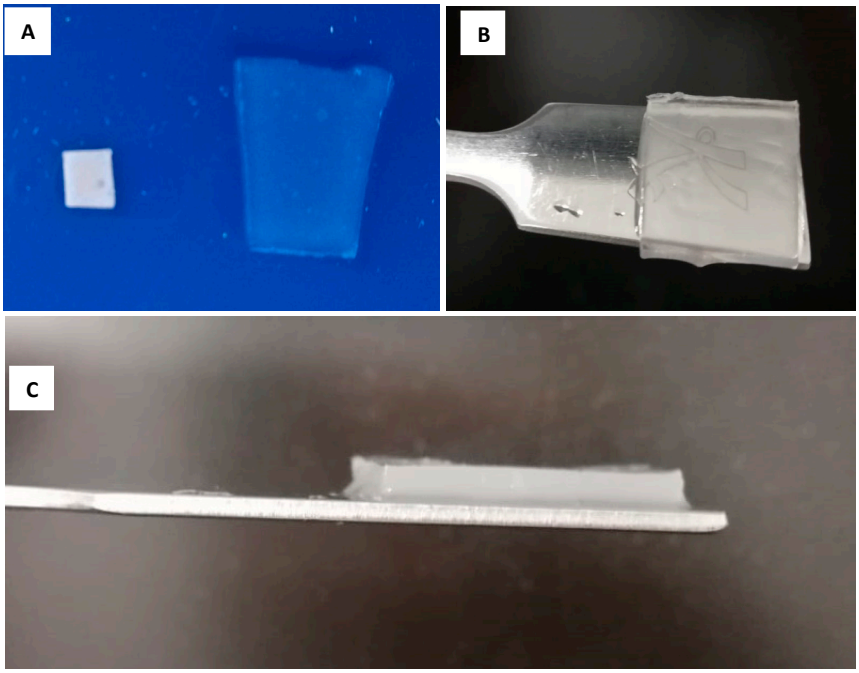

Figure 2. Images showing (**A**) dry (left) and swollen (right) HA5-PT20 Hydrogel films. (**B**) Closer view of a swollen HA5-PT20 Hydrogel film, and (**C**) side view of the thickness of the swollen HA5-PT20 hydrogel film.

3.2. Effect of pH on Hydrogel Film Formation

Preliminary results showed that when HA and PT were mixed in distilled water before being casted to a xerogel film for crosslinking, there was no swelling observed in the films regardless of the

cross-linking method. These films dissolved after immersion in the swelling medium, which indicates no reaction between HA and PT and, therefore, shows an absence of cross-linking.

As such, we then investigated if the pH of the initial HA-PT solution had an effect on the outcome of the cross-linking reaction. The mixing of HA with PT was performed under acidic, neutral, and alkaline pH conditions (Table 1). Acidic and neutral conditions failed to produce cross-linked HA-PT films regardless of PT content and cross-linking technique (UV, microwave, and oven). The cross-linking reaction was effective only when the HA-PT xerogel was casted from an alkaline (pH 11) aqueous mixture (Table 2).

Failure of hydrogel formation under acidic and neutral pH conditions indicated that the reactants (HA and PT) require a source of hydroxyl anions (O^-) to facilitate their reactivity. In that case, an acidic condition (hydrogen ions, H^+) hindered the activation of the two reactants. It is plausible that PT initiates the reaction since it has two pairs of electrophilic carbonyl carbons in the tetra-acrylate group. The acrylate moiety in the PT is typically acidic by nature and, therefore, can be activated by hydroxide anions generated from alkaline conditions. Alkaline pH generates enough O^-, which are nucleophiles that attack the electrophilic carbonyl carbons of the PT to form an OH group. This, subsequently, attacks the electrophilic carbonyl carbon of the HA, which has a -OH as a leaving group.

3.3. Cross-Linking Method

3.3.1. Effect of UV-Irradiation Time

When the amount of PT in the xerogel was 10% w/w (HA5-PT10) and 15% w/w (HA5-PT15), there was insufficient cross-linking and swelling of the xerogels. It was observed that a PT concentration of 20% w/w and above was required for sufficient cross-linking of the HA using the UV irradiation method.

The swelling and cross-linking behaviour of the films containing 20% w/w PT (HA5-PT20) is shown in Table 3. The percentage of swelling increased with UV irradiation time from 5692 ± 493% at 10 min to 11894 ± 745% at 40 min, which indicates that the shorter the UV irradiation time is, the higher the degree of cross-linking attained in the films is. All swelling parameters were in agreement [10]. For example, increasing UV-irradiation time decreased the percent of gel fraction (the insoluble part of the dried sample following hydration), with the lowest 69.22 ± 6.50% being observed at 20 min. This observation supports that excessive UV irradiation destroys initially formed cross-links in the films. A lower volumetric swelling ratio (Qv) indicates a higher crosslink density [12]. Qv increased with UV-irradiation time from 126 ± 10.69 at 10 min to 264 ± 16.65 at 40 min. This was also the case with Mc (MW between crosslinks), which supports that prolonging UV-irradiation was disadvantageous to crosslinking in HA5-PT20 hydrogel films. The lower the swollen hydrogel mesh size (ξ) and effective crosslink density (Ve) are, the higher the cross-linking in the hydrogel film is [12]. ξ increased with irradiation time from 1699 ± 172 at 10 min to 4014 ± 292 at 40 min. Ve also increased with increasing irradiation time with maximum Ve (6.41×10^{-7}) observed at 40 min of UV irradiation time.

Table 3. Swelling, mesh size, and cross-linking behaviour of HA5-PT20 hydrogels crosslinked by the UV irradiation method.

UV Time (min)	Swelling (%)	Equilibrium Swelling (%)	Gel Fraction (%)	Qv	Mc g/mol	Mesh Size (ξ) nm	Crosslinking Density (Ve) mol/cm^3
10	5692 (±493)	98.26 (±0.14)	71.39 (±4.77)	126 (±10.69)	3.74×10^6	1699 (±172)	3.31×10^{-7}
20	5979 (±647)	98.28 (±0.17)	79.45 (±2.85)	128 (±13.65)	3.86×10^6	1733 (±215)	3.24×10^{-7}
30	8670 (±1984)	98.48 (±0.25)	78.96 (±4.08)	192 (±44.41)	4.38×10^6	2779 (±751)	1.75×10^{-7}
40	11,894 (±745)	99.16 (±0.05)	69.22 (±6.50)	264 (±16.65)	1.27×10^7	4014 (±292)	6.41×10^{-7}

3.3.2. Microwave Method

The microwave method achieved the formation of cross-links in films containing PT concentrations from 10% w/w and above (Tables 4–7).

The percentage of swelling was gradually decreasing with increasing microwaving time from 10 min onward, which indicates increasing formation of cross-links up to 30 min. Beyond that time, there was a plateau or slight compromise of the crosslinks. Films containing 10 %w/w PT (HA5-PT10) failed to cross-link after 10 min microwaving but showed a gradual and time-dependent crosslinking from 20 min onward (Table 4).

The percentage of swelling was significantly higher in HA5-PT10 compared to HA5-PT20 films ($p = 0.0015$), which indicates a concentration dependence of PT on crosslinking. One-way analysis of variance (ANOVA) revealed significant differences among HA5-PT20, HA5-PT15, and HA5-PT10 hydrogels in terms of the percentage of swelling ($p < 0.05$), equilibrium swelling percentage ($p = 0.0001$), percentage of gel fraction ($p = 0.0074$), Qv ($p < 0.05$), Mc ($p = 0.0026$), ξ ($p < 0.05$), and Ve ($p = 0.0098$) (Tables 4–6).

In contrast to the above trend, films containing 30% w/w PT (HA5-PT30) exhibited a higher percentage of swelling and lower gel fraction compared to the rest (Table 7). This observation shows that excess amount of PT in the film can compromise its ability to cross-link with HA and that 20% w/w was the maximum acceptable concentration of PT in the film.

A comparison between the microwave and UV methods showed a significantly higher gel fraction percentage ($p = 0.0094$), Ve, and ξ ($p < 0.05$) for the microwaving method.

Table 4. Swelling, mesh size, and cross-linking behaviour of HA5-PT10 hydrogels crosslinked by the microwave method.

Microwave Time (min)	Swelling (%)	Equilibrium Swelling (%)	Gel Fraction (%)	Qv	Mc g/mol	Mesh Size (ξ) nm	Crosslinking Density (Ve) mol/cm^3
10	–	–	—				
20	8593 (±266)	98.84 (±0.03)	63.04 (±1.57)	191 (±5.56)	7.43×10^6	2746 (±99.41)	1.65×10^{-7}
30	7737 (±328)	98.69 (±0.05)	79.13 (±3.75)	168 (±7.09)	6.01×10^6	2369 (±119.9)	3.06×10^{-7}
40	6370 (±305)	98.44 (±0.07)	80.38 (±2.75)	141 (±6.80)	4.51×10^6	1937 (±108.4)	2.72×10^{-7}
60	5700 (±599)	98.25 (±1.88)	83.55 (±3.67)	126 (±13.2)	3.76×10^6	2283 (±207.8)	3.32×10^{-7}

Table 5. Swelling, mesh size, and cross-linking behaviour of HA5-PT15 hydrogels crosslinked by the microwave method.

Microwave Time (min)	Swelling (%)	Eq. Swelling (%)	Gel Fraction (%)	Qv	Mc g/mol	Mesh Size (ξ) nm	Crosslinking Density (Ve) mol/cm^3
10	7968 (±791)	98.74 (±0.11)	70.15 (±1.13)	177 (±17.43)	6.57×10^6	2516 (±292)	1.89×10^{-7}
20	4094 (±532)	97.32 (±0.75)	82.43 (±1.36)	85 (±21.37)	1.97×10^6	1073 (±313)	3.78×10^{-6}
30	3755 (±536)	97.49 (±0.54)	89.22 (±3.20)	83 (±12.28)	1.88×10^6	1046 (±174)	6.75×10^{-7}
40	4037 (±958)	97.25 (±0.99)	94.87 (±2.82)	89 (±21.07)	2.15×10^6	1142 (±312)	6.40×10^{-7}
60	4612 (±571)	97.85 (±0.28)	92.90 (±1.77)	102 (±12.42)	2.64×10^6	1330 (±190)	4.77×10^{-7}

Table 6. Swelling, mesh size, and cross-linking behaviour of HA5-PT20 films crosslinked by the microwave method.

Microwave Time (min)	Swelling (%)	Eq. Swelling (%)	Gel Fraction (%)	Qv	Mc g/mol	Mesh size (ξ) nm	Crosslinking Density (Ve) mol/cm^3
10	7763 (±211)	98.72 (±0.03)	86.57 (±1.85)	172 (±5.13)	6.27×10^6	2440 (±77.9)	2.62×10^{-7}
20	2341 (±342)	95.84 (±0.62)	95.35 (±1.46)	51 (±7.76)	5.48×10^5	603 (±102.6)	1.49×10^{-6}
30	1171 (±31)	92.12 (±2.34)	95.82 (±0.17)	19 (±5.50)	1.73×10^5	195 (±64.6)	4.96×10^{-6}
40	1216 (±180)	92.29 (±1.28)	97.95 (±1.73)	26 (±4.16)	2.88×10^5	281 (±48.2)	4.45×10^{-6}
60	1267 (±196)	92.57 (±1.46)	90.93 (±0.92)	25 (±5.85)	2.70×10^5	267 (±67.7)	4.90×10^{-6}

Table 7. Swelling, mesh size, and cross-linking behaviour of HA5-PT30 hydrogels crosslinked by the microwave method.

Microwave Time (min)	Swelling (%)	Eq. Swelling (%)	Gel Fraction (%)	Qv	Mc g/mol	Mesh Size (ξ) nm	Crosslinking Density (Ve) mol/cm^3
20	9158 (±595)	98.91 (±0.07)	77.45 (±3.11)	203 (±13.5)	8.27×10^7	2959 (±224)	1.49×10^{-7}
30	8806 (±591)	98.87 (±0.07)	79.24 (±3.54)	195 (±13.3)	7.75×10^7	2827 (±221)	1.59×10^{-7}
40	7198 (±397)	98.62 (±0.06)	75 (±3.57)	160 (±8.88)	5.53×10^7	2234 (±144)	2.22×10^{-7}
60	8121 (±584)	98.77 (±0.08)	79.44 (±4.19)	180 (±12.8)	6.77×10^7	2572 (±216)	1.82×10^{-7}

3.3.3. Oven Method

Swelling and cross-linking parameters of the HA5-PT30, HA5-PT20, HA5-PT15, and HA5-PT10 films crosslinked in the oven for 24 h via thermal treatment at 80 °C are shown in Table 8.

Table 8. Swelling, mesh size, and cross-linking behaviour of HA5-PT hydrogels crosslinked by the oven-assisted thermal method (24 h).

Hydrogel Name	Swelling (%)	Eq. Swelling (%)	Gel Fraction (%)	Qv	Mc g/mol	Mesh Size (ξ) nm	Crosslinking Density (Ve) mol/cm^3
HA5-PT30	7683 (±1053)	98.69 (±0.15)	81.98 (±2.65)	171 (±23.3)	6.20×10^7	7239 (±388)	2.03×10^{-7}
HA5-PT20	1806 (±456)	94.54 (±1.33)	101.23 (±0.47)	40 (±10)	5.65×10^5	446 (±130.5)	2.45×10^{-6}
HA5-PT15	5848 (±785)	98.29 (±0.21)	102.63 (±2.73)	129 (±17.21)	3.93×10^6	1755 (±276)	3.21×10^{-7}
HA5-PT10	8101 (±174)	98.77 (±0.02)	87.30 (±1.58)	180 (±4.00)	6.73×10^6	2564 (±64.04)	1.82×10^{-7}

Similar to the microwaving method, there was a correlation between increasing PT concentration and cross-linking efficacy up to 20% w/w PT. HA5-PT20 was the optimum formulation as it exhibited the lowest percentage of swelling and equilibrium percentage of swelling, Qv, and ξ, with the highest gel fraction and Ve.

The microwaving and oven methods showed comparable crosslinking efficiency and were superior to the UV irradiation method, as deduced by the significantly lower percentage of swelling for the HA5-PT20 films.

3.4. Cross-Linking Mechanism

PT has previously been used as a crosslinker of polyethylene oxide (PEO) hydrogel films synthesised via UV-radiation [10]. An increase in PT concentration was shown to significantly increase the gel fraction and crosslinking density, while causing a significant decrease in equilibrium water capacity, overage MW between cross-links, and mesh size [10]. According to Wong et al. [10], when PT absorbs energy from UV photons, its acrylate group generates reactive-free radicals, which initiates the cross-linking of repeated units of PEO with PT radicals via recombination. Similarly, it is possible that UV photons generate PT radicals, which abstracts a hydrogen atom from HA to generate HA radicals for repeated HA units that recombine with PT radicals to form crosslinks (Figure 3). The cross-linking of PT and HA radicals could be via the hydroxyl (-OH) group of the HA and the carboxylic (-COOH) group of the PT (Figure 4). In the present study, when PT was activated by -OH as nucleophiles generated under alkaline conditions, they generate radicals, which activate radical polymerization.

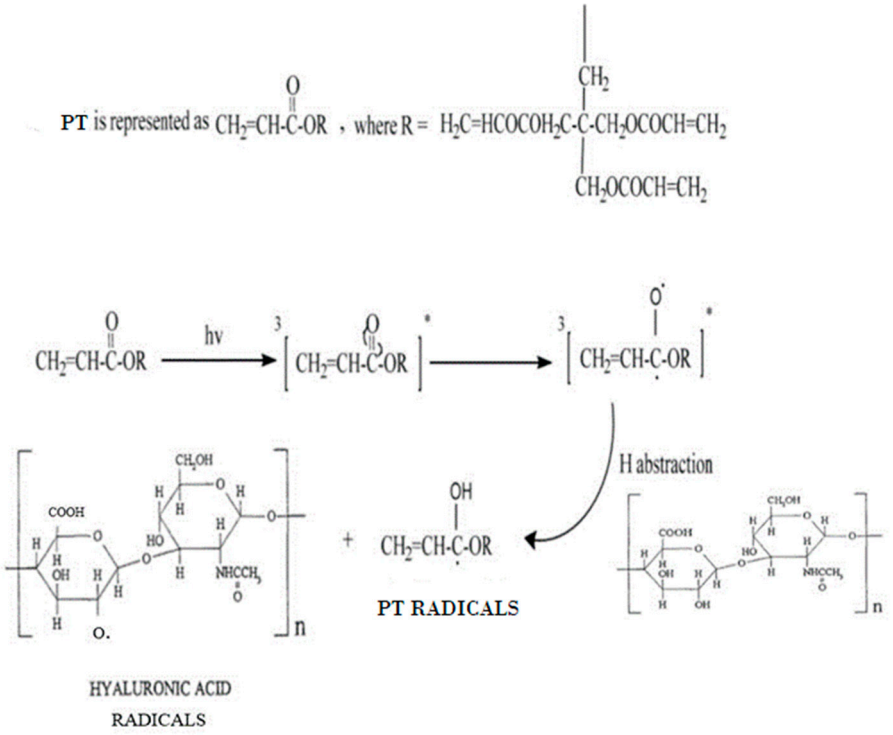

Figure 3. Generation of reactive HA free radicals with the aid of PT as photo-initiator [10].

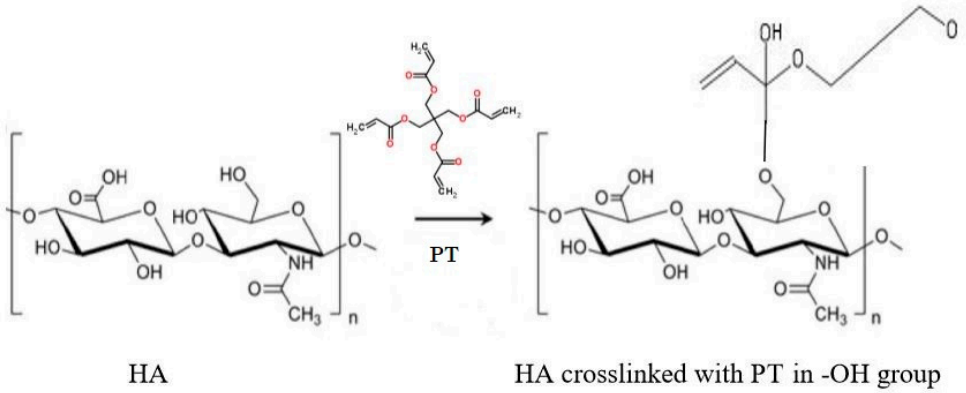

Figure 4. Crosslinking between HA and PT polymers via the -OH group of the HA and carbonyl carbon of the PT.

3.5. FTIR Spectral Analysis

The FTIR spectra of pure HA, cross-linked HA5-PT20 xerogel film, and pure PT are shown in Figure 5. It can be observed that peaks present in the spectra of pure HA were all present in the spectra of the crosslinked HA5-PT20 xerogel film. However, not all peaks of the pure PT were present in the spectra of the crosslinked HA5-PT20 film. Notably, C=O groups, represented by a broad stretching vibration peak at around 1738 cm^{-1} in pure PT, were reduced in the cross-linked HA5-PT20 hydrogel film. This indicates that the carbonyl carbon in the PT reacted with the hydroxyl group of the HA to form methyl vinyl ether unit (C-O-C), which had a stretching vibration peak at around 1100–1300 cm^{-1}. The C=C group stretch in the range of 1600–1680 cm^{-1} indicates that the unsaturated alkene bond of the PT was not destroyed in the cross-linked HA5-PT20 hydrogel film [14,15]. Therefore, a possible cross-linking mechanism would be the formation of an ester bond (C-O-C) between the hydroxyl group of the HA and the carbonyl carbon of the PT.

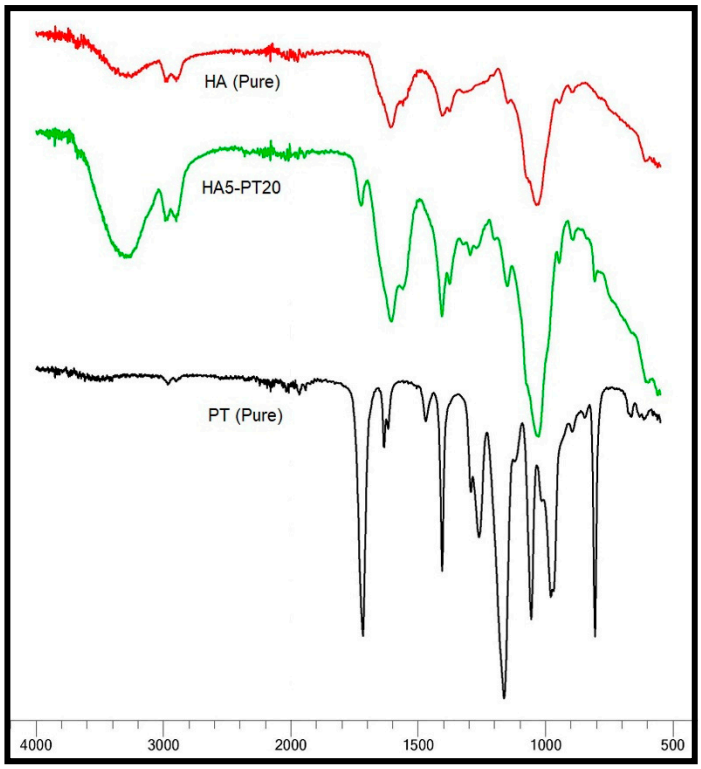

Figure 5. FTIR spectra of pure HA, cross-linked HA5-PT20, and pure PT.

The FTIR spectra of HA5-PT xerogels cross-linked by the different methods were recorded and compared to study the chemical interactions and crosslinking mechanisms. Comparison between the spectra of the HA5-PT30 crosslinked by UV irradiation (10 min) and the non-crosslinked (NC) control film (Figure 6) indicated the presence of a newly introduced ester bond (C-O-C) with a peak at around 1180 cm^{-1} for the cross-linked film. This confirms the cross-linking mechanism by formation of an ester bond. However, the degree of cross-linking was low due to excess PT. A brief UV-irradiation of HA5-PT20 exhibited a better cross-linking than prolonged irradiation (Figure 7), which confirms the observation from the swelling results that prolonged UV-irradiation destroyed the cross-links and, hence, impeded swelling.

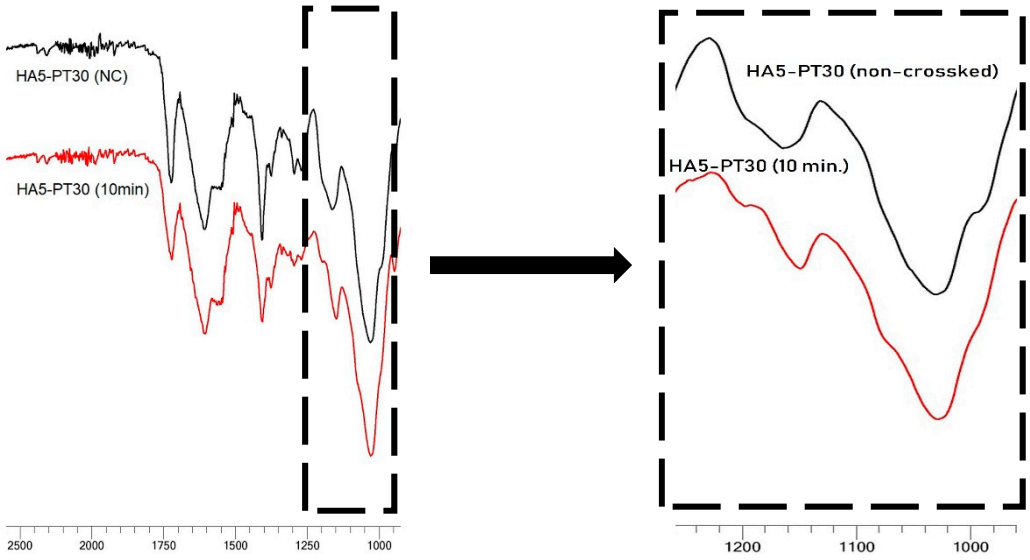

Figure 6. HA5-PT30 crosslinked by UV irradiation (x-axis is the wavenumber/cm^{-1}).

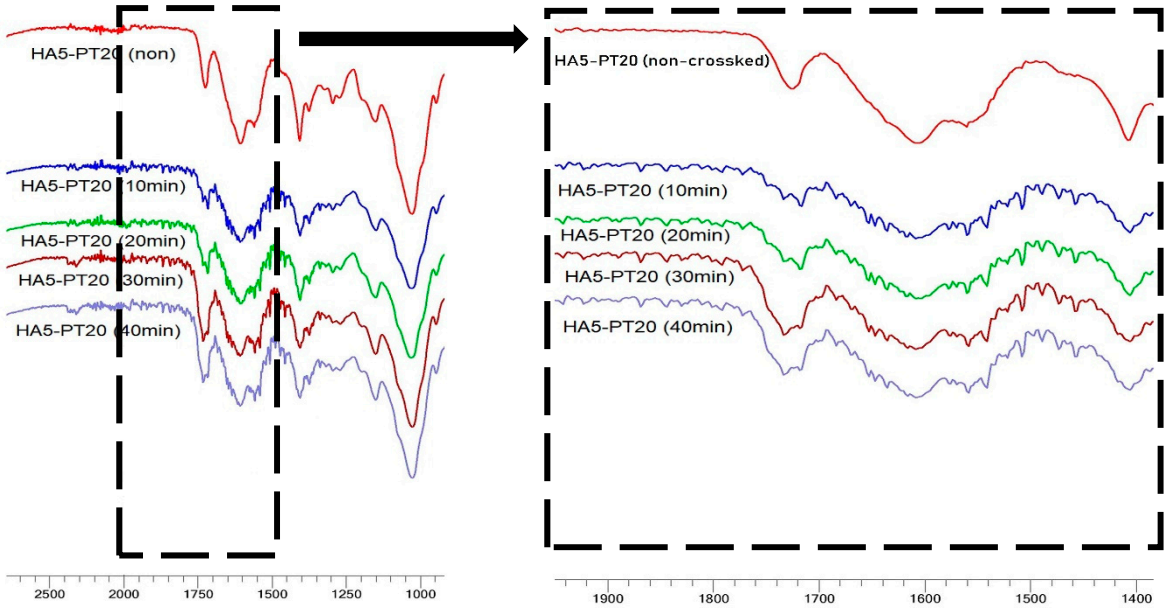

Figure 7. HA5-PT20 crosslinked by UV irradiation (x-axis is the wavenumber/cm^{-1}).

The FTIR data for the microwave cross-linked HA5-PT samples was in congruence with their swelling data. HA5-PT30 exhibited poor cross-linking due to excess PT (Figure 8). HA5-PT20 was the best mix, with an optimum microwave irradiation time of 30 min, which preserved the C=C in the acrylate group at around 1640 cm^{-1}. Prolonged microwaving resulted in loss of the acrylate. Therefore, excessive swelling was observed (Figure 9). When the amount of PT was reduced to HA5-PT15 and further to HA5-PT10 (Figures 10 and 11), the hydrogels exhibited poor cross-linking, which leads to a higher percentage of swelling.

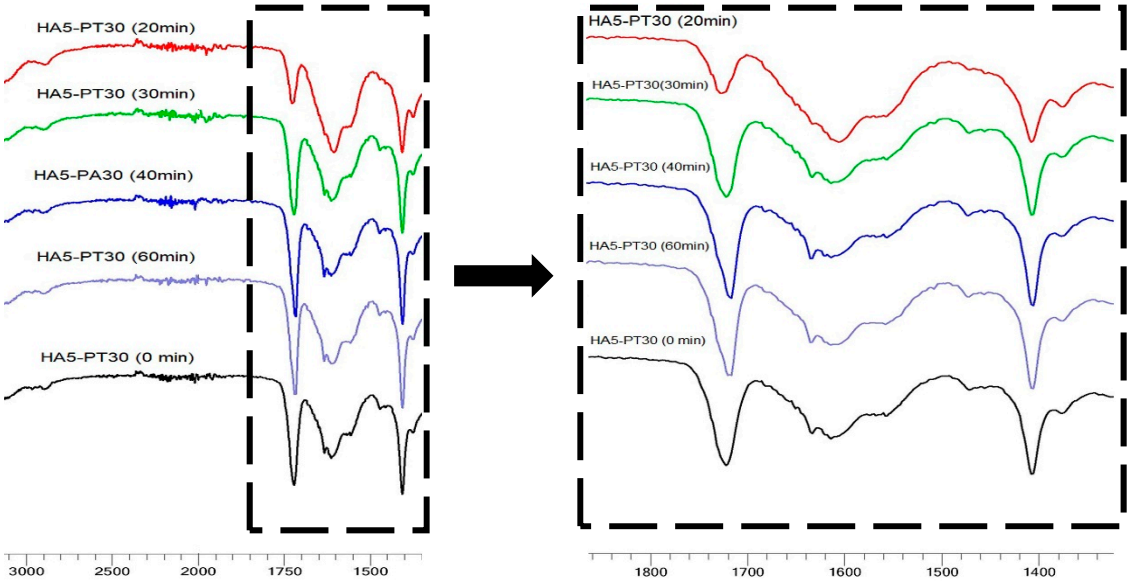

Figure 8. HA5-PT30 crosslinked by microwave irradiation (x-axis is the wavenumber/cm^{-1}).

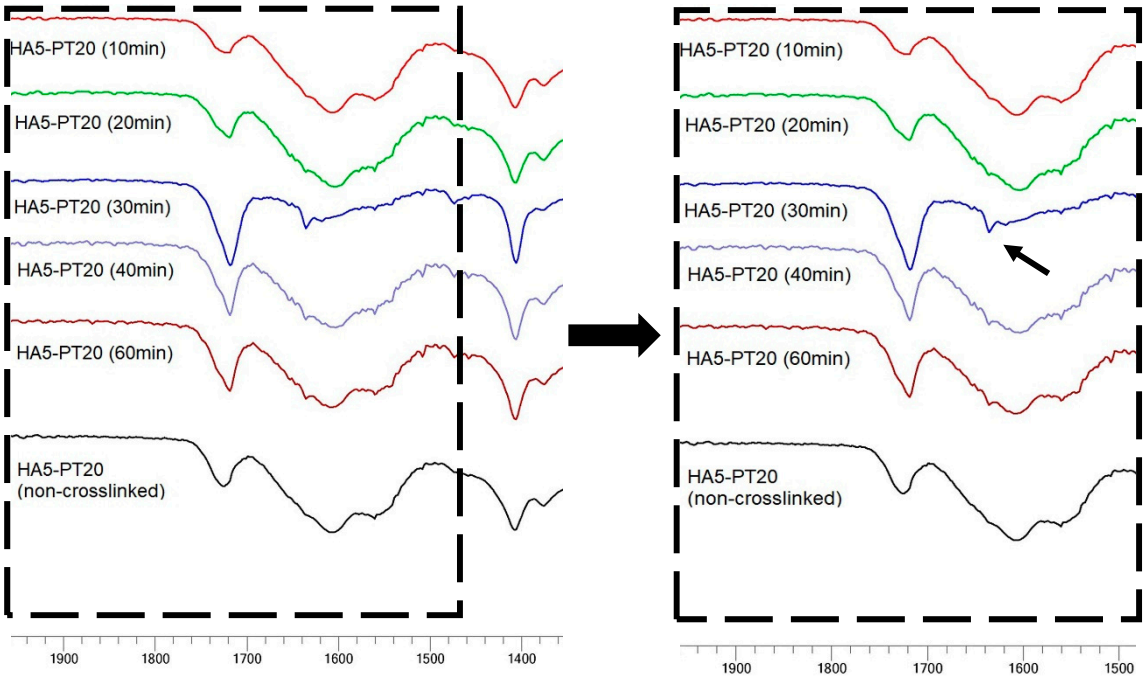

Figure 9. HA5-PT20 crosslinked by microwave irradiation (x-axis is the wavenumber/cm^{-1}). At 30 min, the C=C in the acrylate group, which is around 1,640 cm^{-1} was preserved.

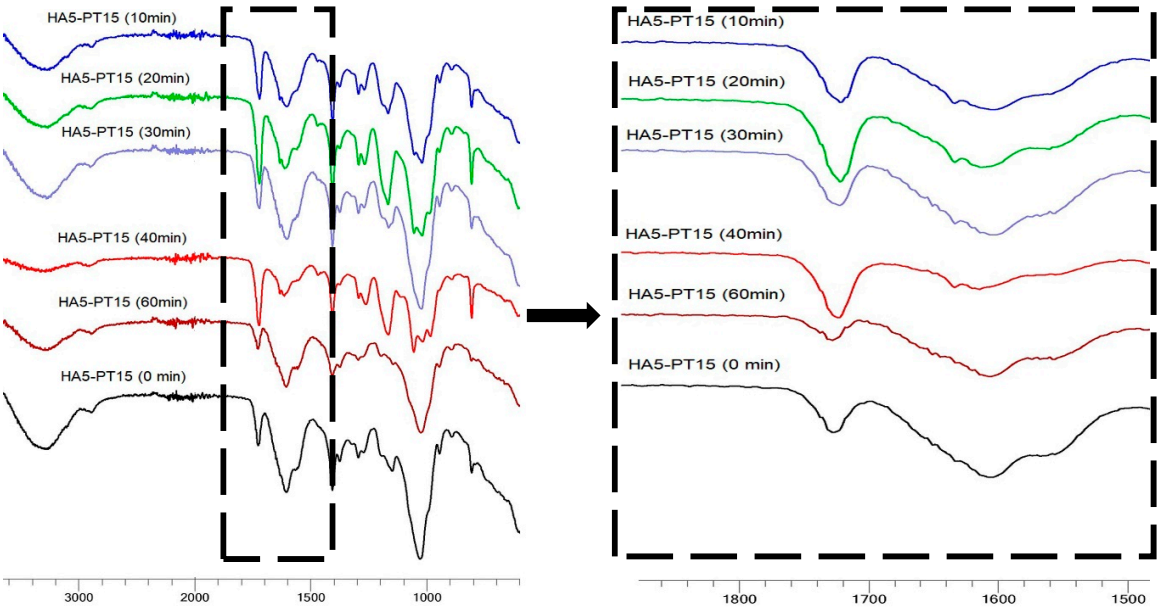

Figure 10. HA5-PT15 crosslinked by microwave irradiation (x-axis is the wavenumber/cm^{-1}).

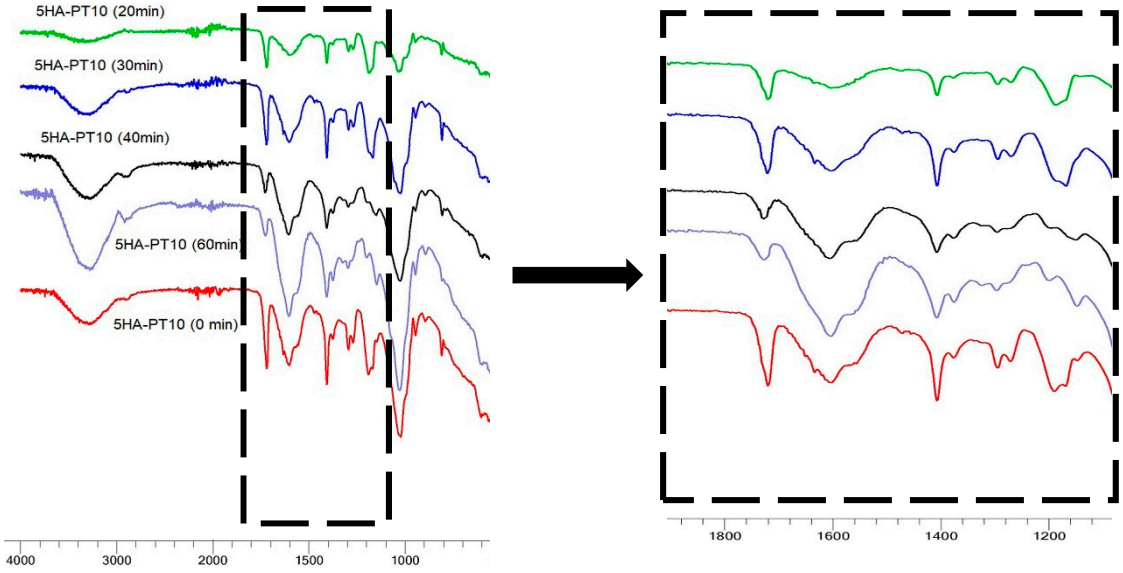

Figure 11. HA5-PT10 crosslinked by microwave irradiation (x-axis is the wavenumber/cm^{-1}).

FTIR results were also in agreement with the swelling data demonstrating that oven-assisted cross-linking was better than the UV-irradiation method. The FTIR spectra of HA5-PT30 (Figure 12), show that oven heating preserved the C=C in the acrylate group, which is around 1640 cm^{-1}. HA5-PT20 was the best formulation since it exhibited a diminished C=O stretching vibration peak at 1738 cm^{-1} and an ester bond (C-O-C) with a peak at around 1180 cm^{-1} indicative of the cross-link bond (Figures 13–15). When the amount of PT was reduced to HA5-PT15 (Figure 16) and HA5-PT10 (Figure 17), the hydrogels exhibited poor cross-linking behaviour and, hence, a higher percentage of swelling.

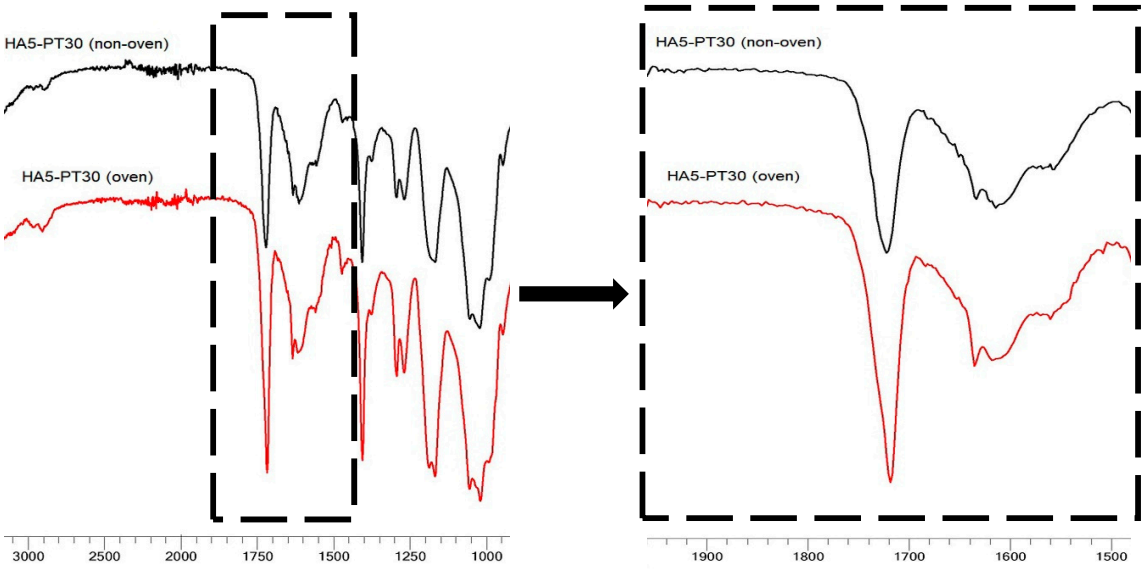

Figure 12. HA5-PT30 crosslinked by oven-assisted thermal method (x-axis is the wavenumber/cm^{-1}).

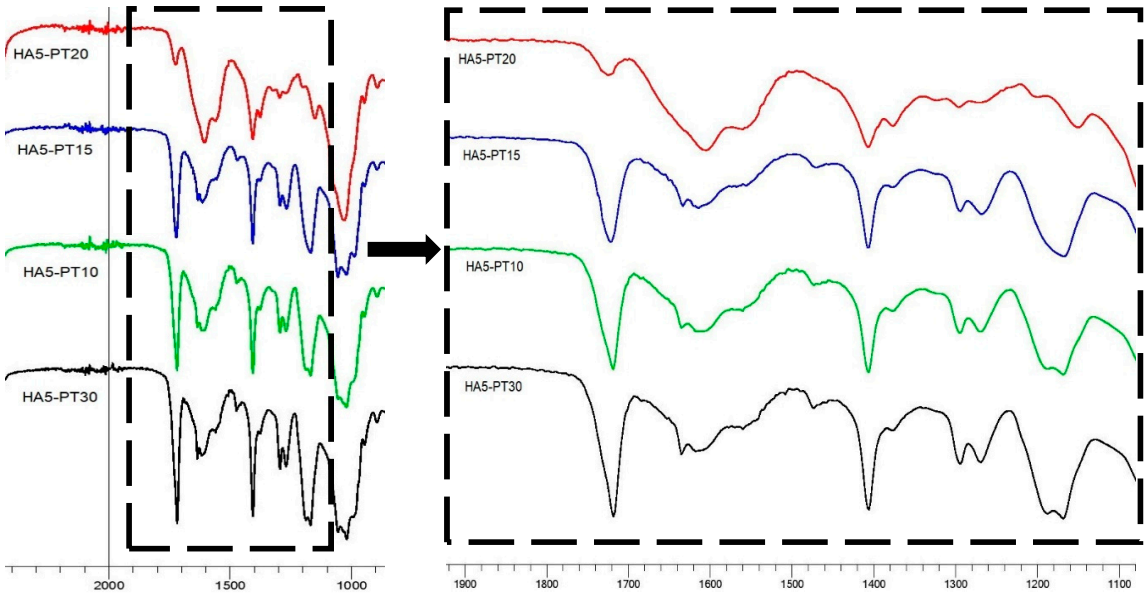

Figure 13. HA5-PT crosslinked by oven-assisted thermal method (x-axis is the wavenumber/cm^{-1}).

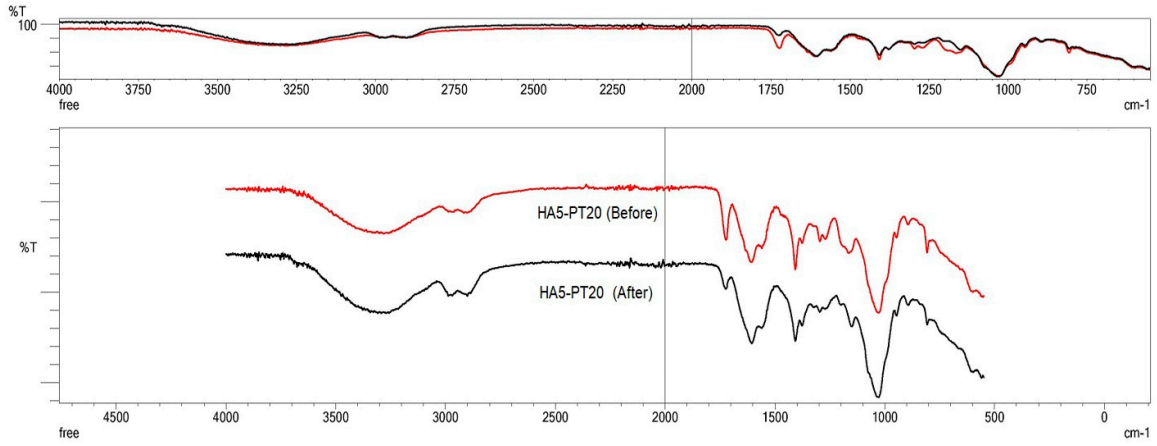

Figure 14. HA5-PT20 before and after crosslinking by oven-assisted thermal method (x-axis is the wavenumber/cm^{-1}).

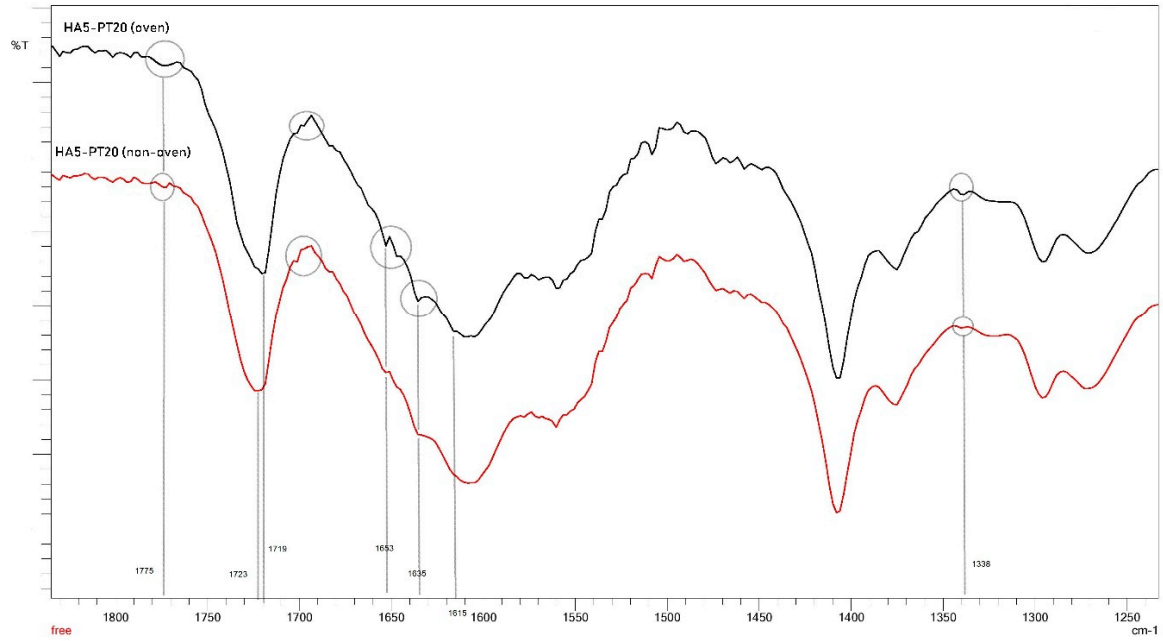

Figure 15. HA5-PT20 before and after crosslinking by the oven-assisted thermal method (x-axis is the wavenumber/cm^{-1}).

Overall, our novel cross-linked film of HA is aimed to be advantageous toward the delivery of cosmetic and/or pharmaceutical actives. In previous studies, we found that cross-linked hydrogel patches of PEO showed sustained release of the loaded actives [16]. Therefore, we now explored the use of HA as the polymer material for the synthesis of the cross-linked hydrogel platform. A potential limitation of our novel films can be the presence of residual PT monomer units that could be sensitized to the skin. Residual (*ie* uncrosslinked) PT could diffuse via the skin due to its low MW. Therefore, it is important to conduct residual content analysis for PT in the optimised hydrogel films and, subsequently, to adjust the PT amount in the formulation if necessary, so as to ensure that there is no unreacted PT and to establish the biocompatibility of our novel hydrogel films. We will conduct this analysis using the method we previously developed for the quantification of residual PT in PEO hydrogel films [17]. Toxicological studies of PT and similar crosslinking agents on guinea pigs showed that PT had a lower sensitising effect [18].

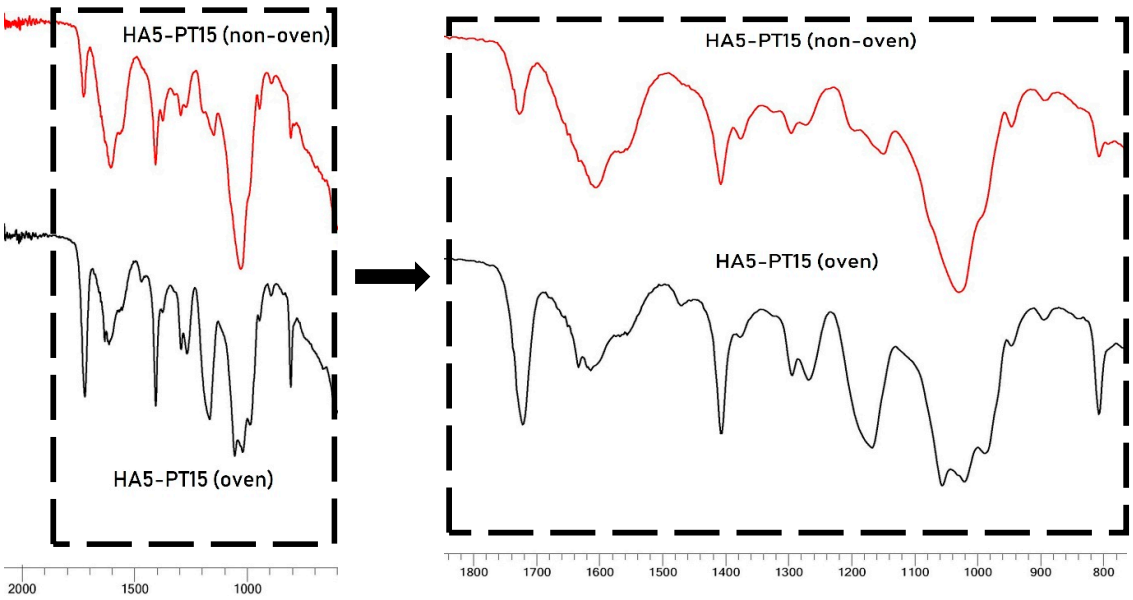

Figure 16. HA5-PT15 before and after crosslinking by the oven-assisted thermal method (x-axis is the wavenumber/cm^{-1}).

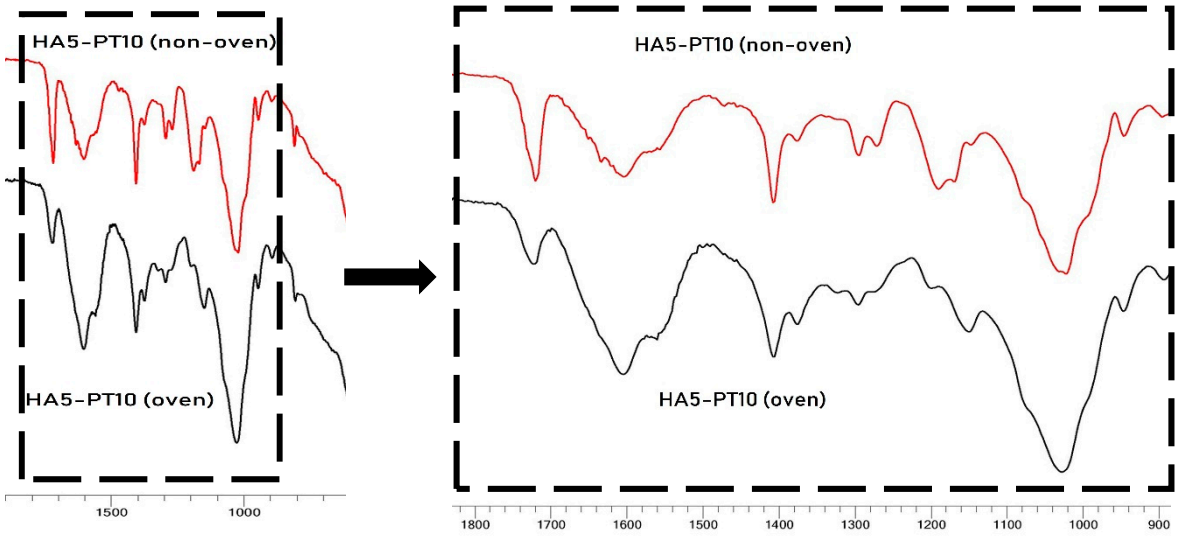

Figure 17. HA5-PT10 before and after crosslinking by the oven-assisted thermal method (x-axis is the wavenumber/cm^{-1}).

4. Conclusions

In this study, we report the successful synthesis of novel cross-linked HA hydrogel films using PT as the cross-linking agent. Cross-linking was achieved when mixing the HA and PT under alkaline conditions but not under acidic or neutral. The optimum formulation and cross-linking method were HA5-PT20 (20% w/w PT in the film) using the oven method for 24 h or microwave method for 30 min. The UV method was disadvantageous as it destroyed the cross-links in the hydrogel films leading to an increased percentage of swelling. The microwave method showed reproducible and consistent cross-linking but, due to uneven radiation, some of the films tended to get burnt at their edges.

Therefore, the oven method was found to be superior for the cross-linking of HA-PT hydrogel films due to its safety, simplicity, uniformity of treatment, and ability to process larger batches compared to the UV-irradiation and microwave methods.

The next steps after this study are the characterisation of the optimised HA-PT hydrogel films including their toxicological safety evaluation via residual PT content analysis, and then studies on the loading of cosmetic ingredients.

Author Contributions: Conceptualization, K.D. and F.R. Data curation, K.D. and F.R. Formal analysis, F.R. and M.A. Investigation, F.R. Methodology, K.D. and F.R. Project administration, K.D. Resources, K.D. Supervision, K.D. Validation, K.D. Visualization, K.D., F.R., and M.A. Writing—original draft, F.R. and M.A. Writing—review & editing, K.D.

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Conflicts of Interest: The authors declare no conflict of interest.

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