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Influence of photoinitiator concentration and irradiation time on the crosslinking performance of visible-light activated pullulan-HEMA hydrogels

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Abstract:

In-situ forming hydrogels were prepared from pullulan-HEMA copolymer using three-component visible-light system composed of camphorquinone carboxylic acid-folic acid-iodonium salt. The relevance of double bond conversion and crosslinking density of hydrogels with the photoinitiator concentration and irradiation time were estimated by FT-IR analysis and swelling calculation using Flory-Rehner theory, respectively. The results revealed that the crosslinking density and degree of conversion of hydrogels were improved by photoinitiator concentration increasing until certain extend, then they decreased due to a primary radicals termination reaction occurred. The shortest irradiation time of 10s was essential to obtain acceptable hydrogels for further characterizations. For the probability use of hydrogels as scaffold was investigated *in vitro* by measuring of the indirect cytotoxicity assay by MTT-assay using human bone *Sarcoma* cell as a reference cell lines. The majority of seeded SW1353 cells maintained a live with an accepted viability of ~ 85-92% over a four days culture period with irradiation of hydrogel 10 s, while cell viability has improved to ~95-98% with prolonging the irradiation time of hydrogel to 60s. The current photoinitiating system is a proper system for *in-situ* crosslinking the activated-light biomaterials for bone regeneration, dental, or tissue engineering applications.

Keywords: Pullulan-HEMA; Carboxylated camphorquinone; Folic acid

1. Introduction

Photopolymerization is widely preferred because hydrogels can be obtained at temperature and pH conditions close to physiological medium with presence of biologically active molecules [1, 2]. In spite of previous reports of photopolymerizable hydrogels for biomedical applications, two limitations are still addressed gradually by researchers [2]. First, the possible toxicity issue of the used photopolymerization system ingredients [1]; second, the harmfulness issue of the used light-irradiation source either UV or γ-rays [2]. The visible-light induced photopolymerization technique using blue-light absorbed photoinitiator *type II*, has many advantages such as its flexibility for hydrogels preparation, and it's easy and more concise method for drug-loading

compared to other polymerization methods [3]. In addition, visible light is known to have less damage effect to cells and has efficiently transmitting through tissues, resulting strong deep curing. If the aforementioned limitations have been overcome and entirely addressed, the photo-crosslinked hydrogels for biomedical applications could be strongly grown and developed. In our previous work on photopolymerization for vinyl monomers, dextran-HEMA hydrogels have been crosslinked under visible-light induced system using camphorquinone (CQ) as photoinitiator and amines coinitiator (*e.g.,* DMAEMA, NPG, or BDO) [4]. In that study, the hydrogel formation showed some difficulties *e.g.* poor-water solubility of CQ photoinitiator and acute toxicity of amines coinitiator. Thus, lots of efforts and attempts have been done to develop a new water-soluble and visible-light absorbed photoinitiator. Two-component photoinitiating system under visible-light composed of riboflavin as photoinitiator and *L*-arginine as coinitiator was used for crosslinking dextran-methacrylate hydrogels [5]. This system offered a long irradiation time ranged between (15-40 minutes), and a weak photo-bleaching was observed due to riboflavin is a mainly producing yellow dye existing in many plants and microorganisms [5]. In addition, Arakawa *et al.* [6] has used riboflavin for crosslinking glycol-chitosan-GMA-collagen hydrogels under visible light for bone tissue engineering.

Recently, Hydroxyethyl starch-HEMA hydrogels have been photocrosslinked under visible-light using three-component photopolymerization system composed of carboxylated-CQ (CQCOOH) as photoinitiator, DMAEMA as coinitiator, and DPIC as accelerator [7]. This system presented successful and efficient photoinitiating system in terms of its short irradiation time at 5 seconds, very strong photo-bleaching, high water solubility and non-toxicity of photoinitiator [7]. On the contrary, both DMAEMA amine coinitiator and DPIC accelerator presented toxicity by MTT and LDH assays [6]. According to last contributions and studies, we have focused on how to develop a new photoinitiating system avoiding all last mentioned problems for biomedical applications. Carboxylated camphorquinone (CQCOOH) is a photoinitiator type II that demands an electron donor to create a free radical upon exposure to visible-light source at wavelength λ_{max} ~ 465 nm [7].

Camphorquinone carboxylic acid (*7,7-Dimethyl-2,3-dioxobicyclo [2.2.1]heptane-1-carboxylic acid*) was first synthesized and known as diketopinic acid for modification of arginine [8]. Hence, CQCOOH was used for the first time as photoinitiator by Ikemura *et al.* [9], while the modified synthetic route for production of CQCOOH has been developed and improved by our

previous study [7]. Amines coinitiators have been widely utilized as electron donors for photoinitiator type II, so this system was known as a ketone-amine initiation system. Encinas *et al.* [10] have reported the effect of amine structure and type on the polymerization efficiency under UV-light irradiation source. Similarly, Kamoun and Menzel [4, 11] demonstrated the effect of amines (*e.g.* DMAEMA and NPG) and non-amines (*e.g.* BDO) coinitiators' type on the crosslinking density for dextran-HEMA and HES-HEMA hydrogels under visible-light irradiation. Exclusively, the folic acid is employed for the first time as a safe and alternative/effective coinitiator instead of amine coinitiators. Meanwhile, the iodonium salt derivative *e.g.* diphenyliodonium tetrafluoroborate (DPITFB) is used herein for the first time in the current photopolymerization system too, as accelerators to regenerate the dye of initiator, resulting a free radical formation is sharply improved and produces additional active radicals [4,7,11,12].

This work aims to evaluate the photocrosslinking performance of pullulan-HEMA hydrogels using CQCOOH-folic acid-DPITFB system under visible-light irradiation as a new photo-initiating system used in literature. Both the CQCOOH photoinitiator concentration and irradiation time were readjusted to evaluate the polymerization efficiency of the system in terms of the DC% and crosslinking density of formed hydrogels. The optimum concentration of CQCOOH and the shortest irradiation time were determined, while the mechanical properties and cytotoxicity of formed hydrogels were assessed.

2. Materials and methods

2.1. Materials

Pullulan (M_w = *10,000 g/mol*), Hydroxyethyl methacrylate (HEMA), 1,1-Carbonyldiimidazole (CDI), 4-(N,N-Dimethylamino) pyridine (DMAP) and Diphenyliodonium tetrafluroborate (DPITFB, 97.0%) were supplied from Sigma-Aldrich (Steinheim, Germany). 7,7-Dimethyl-2,3-dioxobicyclo[2.2.1]heptane-1-carboxylic acid (carboxylated camphorquinone, CQCOOH) was previously synthesized and described elsewhere in details [7]. Folic acid was taken up from Sigma-Aldrich (St. Louis, MO, USA). Dry/freshly distilled anhydrous tetrahydrofuran (THF) and DMSO were obtained from Fluka Chemie, Germany. Magnesium sulphate (95.0%) and distilled ethyl acetate were obtained from ADWIC Co. for pharmaceutical chemicals, Egypt. Dialysis tubing cellulose membrane (M_{wt} *cut-off 14000*, average diameter 16 mm) was obtained

from Merck, Germany. A LED-lamp (Bluephase, Ivoclar Vivadent, Amhest, NY, USA) was used for irradiation at $\lambda_{max.}$ 460 nm at 1100 mW/cm^2. The irradiation distance was almost 0 cm, while the irradiation time was *ca.* ≥ 30 second.

Fig. 1

2.2. Photocrosslinking of pullulan-HEMA hydrogels under visible light irradiation

Certain degree of substituted pullulan-HEMA copolymers were synthesized and obtained from our previous published work [13]. The pullulan-HEMA copolymer (DS 0.065) was crosslinked under visible-light irradiation using three-component photoinitiating system, consisting of CQCOOH as photoinitiator, folic acid as amine coinitiator, and DPITFB as an accelerator (Fig. 1). Pullulan-HEMA copolymer concentration (20 w/v, %) was dissolved in distilled water for 30 minutes until a homogenous polymer solution was formed, and then the three-component photoinitiating system was added to the mixture as following: 10 mg (0.25 mol%) of CQCOOH photoinitiator was dissolved in polymer solution, and (0.5 mol%) of folic acid was added, moreover (10 mg, 0.5 wt. %) of DPITFB was dissolved in the last mixture. The mixture solution was preserved under gentle moving for 30 minutes at room temperature in the dark-glass bottle for avoiding any premature-polymerization due to the surrounding visible-light. The mixture was poured onto PE molds and photo-crosslinked by LED lamp at zero distance irradiation exposure distance for obtaining (5mm *thick*. and 25mm *diam*.) of hydrogel disk. The hydrogel was formed after less than one minute irradiation time. The gel formation point was found out when a scratch mark remained on the hydrogel surface upon scratching with a spatula. The gelation was complete when the whole gel remained stable without any fluid moving.

2.3. Equilibrium swelling ratio

The known dried masses of crosslinked pullulan-HEMA were soaked in distilled water at fitting time-intervals, and then the samples were taken out. The swollen hydrogels were weighted when the excess of water inters to hydrogel structure. The swollen weights were then compared with their dried weights to calculate the equilibrium swelling ratio (ESR %) when the swollen weight of hydrogel was remained unchanged. ESR% of hydrogels was determined when the weight of swollen hydrogel was remained stable without weight change before the degradation [7].

$$ESR\ \% = (W_s - W_d) / W_d \times 100. \qquad \textit{Eq. (1)}$$

Where W_s and W_d are the weights of hydrogels at the equilibrium swelling state and the dried state, respectively.

2.4. Calculation of crosslinking density and network characteristics of crosslinked pullulan-HEMA hydrogels

The crosslinking density of pullulan-HEMA hydrogels was calculated depending upon the Flory-Rehner formula, where the crosslink density (P_x) is known by the inverse of average number molecular weight between two adjacent crosslinkers (M_c). The M_c can be easily calculated by determination of the swelling ratio based mass, the volumetric swelling ratio at equilibrium swelling state and our previous study [4, 7]. Most hydrogel kinetics of dextran-HEMA crosslinked hydrogels successfully were determined which are similar with pullulan polymer [2].

The crosslinking density $P_x = (M_{c\,v})^{-1}$ *mol* cm^{-3} *Eq. (2)*

Where, $_v$ is the specific volume of dry polymer (0.614 cm^3g^{-1} at 20 ^oC of pullulan) [14].

2.5. Calculation of degree of conversion (DC%) of crosslinked pullulan-HEMA hydrogels

The degree of conversion (*DC %)* of crosslinked pullulan-HEMA hydrogels was calculated by the IR-spectrum integration using "*Essential FTIR® spectroscopy toolbox*" software for data sheet table depending on subtraction of percentage of remained or unchanged C=C at 1650 cm^{-1} after crosslinking process from 100 %.

$DC\ \% = [1- (\Sigma_{C=C\ 1650}/ \Sigma_{C=O\ 1725})] \times 100.$ *Eq. (3)*

Where, the unchanged carbonyl ester group at 1725 cm^{-1} regardless the crosslinking process, was used as internal reference peak [7, 15]. While, the intensity change of the C=C peak at 1650 cm^{-1} of the pullulan-HEMA polymer was employed as an index for the consumption of double bonds, due to the crosslinking process. Therefore, the high intensity of the C=C peak refers to high consumed peaks resulting high DC% value.

2.6. Cytotoxicity test by MTT-assay

Human bone sarcoma cells (SW1353) as reference cell lines were chosen to evaluate the cytotoxicity of photopolymerization system ingredients (*e.g.,* CQCOOH, folic acid, DPITFB, and crosslinked pullulan-HEMA hydrogel disks) using Methylthiazolydiphenyl tetrazolium bromide (MTT) assay. The cells were cultured and grown in RPMI-1640 media supplemented with (10% fetal bovine serum, 10% CO_2, 95% humidity at 37^oC). Briefly, 2×10^5 SW1353 cells ml^{-1} were speared in 200 µl complete media and plated in 96-well micro-plates. The photoinitiating system ingredients and hydrogel pieces were added then cultured and incubated for four days at 37 ºC. The cells were then washed twice with the fresh media after incubation, and 200µl of a tetrazolium salt (0.5 mg/ml PBS) MTT solution was added to each well. After

incubation for another 6 hours at 37°C, the media was left-aside, and the wells were dried. Formazan crystals were re-suspended in 200 µl of DMSO and then shacked for 5 minutes to entirely dissolve formazan in the solvent. The OD was measured at 570 nm with a reference at 630 nm. Another medium containing test substance without cells were measured by the same way to exclude staining affects with adding substances itself [16]. Cell viability percentage was determined, as given in (*Eq. 4*).

$$\textit{Cell viability (\%)} = (A_{test} / A_{control}) \times 100. \qquad \textit{Eq. (4)}$$

Where A_{test} is cells number after incubation and $A_{control}$ is initial cells number before incubation. In case of hydrogel investigation, cells were suspended in polymer solution at the same concentration 2×10^5 cells ml^{-1}, then the mixture was irradiated for 10 and 60 seconds. The hydrogels/cells were cultured in the media as described above. Despite of the free radicals production through the photopolymerization could be accompanied with damage for cultured cells. Thus to evaluate the cytotoxicity effect, cells were encapsulated in hydrogels after various irradiation times using the shortest time possible for sample irradiation.

2.7. Characterizations

FTIR type: (Shimadzu FTIR-8400S, Kyoto, Japan) was used. KBr-sample disk was prepared by crushing polymer sample with infrared grade KBr and then pressing at 105N until getting transparent disk. The FTIR spectrums were obtained by recording 64 scans between 4000-400 cm^{-1} with a resolution of 2 cm^{-1}. FTIR was employed to determine DC % for the double bonds using the changes in the C=C absorbance peak intensity, while unchanged carbonyl ester absorbance as an internal reference, which is only approximate because of the conjugation with the C=C changes the peak shape and intensity as well. The DC was obtained by subtracting the percentage of remaining or uncrosslinked (C=C %) from 100 % [15].

^{1}H-NMR, the proton nuclear magnetic resonance-spectrum was recorded by a NMR-DRX400 instrument with 300 MHz (BRUCKER, Karlsruhe, Germany). Typically, 20 mg of the sample was completely dissolved in 1.0 mL of deuterium oxide NMR-solvent (^2H_2O-d_6), and then the sample was micro-filtered before the measuring.

SEM type: (JEOL, JSM-6360LA, Tokyo, Japan) was used to investigate the interior structure of crosslinked hydrogels. Hydrogel samples were first lyophilized and gold-coated with a sputter coater (model: 11430, USA, connected with vacuum SPi module control model: 11425, USA).

Rheometer type: (*Rheo-Stress* HAAKE *MARS III*, Thermo-Fisher Scientific, USA) was performed to determine the mechanical properties of crosslinked pullulan-HEMA hydrogels. The oscillation shear flow and rotation measurements were performed under the constant temperature at 25^{o}C, using plate-to-plate geometry (PP-20Ti) with angular sweep frequency 0.1-10 Hz. The polymerization system including polymer solution and photoinitiating system was crosslinked *in-situ* the plate, forming hydrogel disk (5mm *thick*. and 25mm *diam*.). The results show the average of three tested samples.

3. Results and discussion

3.1. Synthesis of pullulan-HEMA copolymer

Pullulan-HEMA copolymer was synthesized according to the reported procedure of our previous study for the synthesis of dextran-HEMA [4]. The catalyzed coupling reaction between HEMA-carbonyl imidazolyl and pullulan was proven by FTIR analysis, as shown in (Fig. 2 up). It was noticed that band of a carbonyl ester group at 1725 cm^{-1} which existing clearly only with crosslinked pullulan-HEMA hydrogel, where its absorbance intensity is increased strongly and gradually with crosslinking degree. However, this band neither appears in pure pullulan nor pullulan-HEMA copolymer (*i.e.,* uncrosslinked polymer).

Fig. 2

The successful coupling of HEMA as a side chain in pullulan structure was evidenced and given by ^{1}H-NMR spectra. In spite carbonyl ester group which is responsible to crosslinking performance of pullulan-HEMA could not be detected by NMR spectra; however, this spectrum was utilized to determine the coupling reaction resulting pullulan-HEMA copolymer (Fig. 2, down). In this part, the influence of CQCOOH photoinitiator concentration and irradiation time on crosslinking performance representing on the degree of conversion and crosslinking dentistry of pullulan-HEMA hydrogels is discussed in details.

Fig. 3

3.2. Equilibrium swelling ratio (ESR %)

It is desirable for hydrogel properties to retain its defined structure and to swell adequate amount of water or biological fluids. The ESR of crosslinked pullulan-HEMA hydrogels with various photoinitiator concentrations and various irradiation times was detected for characterizing the stability and water uptake during incubation (Fig. 3). As seen, a minimum gelation time needed for hydrogel formation was 10 s. However, 10 s of irradiation time formed rather weaker gels

due to high swelling ratio, weak crosslinking degree and diffusion of water into a loose network, additionally a long time was required for reaching the equilibrium swelling state (almost 6 days). Comparing with long irradiation times (30, 60, and 120 s) hydrogels which showed a gradual reduction in swelling and short time for reaching equilibrium swelling state (5, 3, and one day) respectively, owing to increasing crosslinking degree with prolonging the irradiation time. Meanwhile, the equilibrium swelling of hydrogels was reduced with photoinitiator concentration from 0.1-0.5 mol% due to the formation of high crosslinked hydrogels, and then it unexpectedly increased with CQCOOH > 0.5 mol% due to the generation of a termination reaction for formed radicals of CQCOOH [4]. Although, highly swollen hydrogels facilitate mass transfer of substances *e.g.,* nutrients, oxygen, and exchange of metabolic waste products in the hydrogels. Also, low crosslinked hydrogels allow to the high amount of water absorption into hydrogel structure lead to an increase in weight loss of hydrogels [4, 7].

Fig. 4

3.3. Effect of CQCOOH photoinitiator concentration on the degree of conversion (%) and crosslinking density of pullulan-HEMA hydrogels

The effect of CQCOOH concentration on the crosslinking performance of pullulan-HEMA hydrogels was shown in Fig. 4 to explain the optimal conditions for the proposed photoinitiating system. Interestingly, only 60 seconds with 0.25 mol % of CQCCOH was a sufficient needed time of irradiation and CQCOOH concentration to initiate the system for crosslinking pullulan-HEMA copolymer. As seen, with a CQCOOH concentration less than 0.1 mol%, no gelation was formed. Notably, the degree of conversion (%) was increased sharply from 62-88% as CQCOOH is increased from 0.1-0.25 mol%, respectively. Then, *DC%* unexpectedly remained almost stable until 0.75 mol% of CQCOOH. Both *DC%* and P_x were significantly reduced with CQCOOH concentration > 0.75 mol%. Similarly, P_x showed crosslinking performance slightly different, where it was increased progressively from 0.1 to 0.75 mol% CQCOOH, then it was pointedly decreased with a CQCOOH concentration > 0.75 mol%. The opposite crosslinking reaction behind a higher CQCOOH photoinitiator contents retarded the crosslinking reaction which might be ascribed to increased the opacity of copolymer solution could obstruct the permeation of free radicals and irradiation light into the solution.

Mostly, the *DC%* and P_x go to increase as the photoinitiator concentration was increased because the greater amount of molecules are available for generation of free radicals. This was probably

owing to the higher free radicals generated as established by the significant correlation between radicals amount and photoinitiator concentration reported by Kamoun *et al.* [7]. As a result, CQCOOH of 0.1-0.75 mol% resulted in a satisfactory hydrogel formation with acceptable form and shape. On the other hand, CQCOOH > 0.75 mol% formed fragile and undesirable mechanical hydrogel, owing to a terminal reaction occurred. Thus, CQCOOH (*i.e.,* 0.25 mol %) is regarded the optimum photoinitiator concentration for this system in terms of the gelation speed and using the little amounts of chemicals as possible to reduce the toxicity issue. However, the relationship between photoinitiator concentration and the polymerization efficiency might be elucidated by an excess of CQCOOH might decrease the DC %. This effect is probably owing to the radiation attenuation during hydrogels by CQCOOH absorption and a phenomenon known as the inner shielding effect, or high rates of primary radical termination which in turn were caused by the high rates of initiation. These observations are consistent with results of Kim *et al*. [5] and Kamoun *et al*. [7], who found that using high photoinitiator concentrations resulted in a terminal reaction for crosslinking of dextran-methacrylate and HES-HEMA hydrogels using riboflavin-arginine and CQCOOH-DMAEMA-DPIC system, respectively.

Fig. 5

3.4. Effect of irradiation time on the degree of conversion (%) and crosslinking density of pullulan-HEMA hydrogels

The free radicals generation is gradually increased with irradiation time prolonging, causing high crosslinked hydrogels [18]. Figure 5 illustrates the influence of several irradiation dose times (10, 20, 30, 60, 120, 180, and 240 seconds) on both the *DC%* and P_x as crosslinking indicators and the extent of photoreactivity of the used polymerization system using 0.25 mol% of CQCOOH concentration. In this system, no hydrogels formed at irradiation time < 10 seconds. Comparing with CQCOOH-DMEMA-DPIC system, the HES-HEMA hydrogels formed at only 5 second due to using a very reactive amine coinitiator, follow-on acute toxicity for the cell proliferation [7]. On the other hand, the current system exhibits a relative long needed irradiation time, despite an expected no toxicity due to using folic acid as primary amine coinitiator. It was found that the P_x is progressively increased with the irradiation time, while the DC % is also increased gradually and reach a maximum value after approximately 120 seconds as irradiation time. Unlike the previous study of CQCOOH-DMAEMA-DPIC system, the maximum P_x of HES-HEMA hydrogel was obtained after irradiation time *ca.* 180 second using the same

CQCOOH concentration (*i.e.,* 0.25 mol%). This explanation indicates that the current initiating system based on CQCOOH-folic acid-DPITFB is higher photo-reactivity than the CQCOOH-amine-DPIC system. Herein, the latter system has a quick response toward the speed-gelation compared to the current system of (CQCOOH-folic acid-DPITFB).

Fig. 6

3.5. Effect of photoinitiator concentration and irradiation time on the morphology of crosslinked pullulan-HEMA hydrogels

The microstructure of crosslinked pullulan-HEMA hydrogels with various photoinitiator concentration and irradiation times are shown in Fig. 6. As ideal scaffolds for tissue engineering application should exhibit a porous structure to allow the nutrients and oxygen for penetrating freely to the inner origins of scaffolds. Thus, the porous structure of scaffolds also should provide the proper position for cell migration and more proliferation. The interior morphology of lyophilized crosslinked pullulan-HEMA hydrogels was characterized by SEM and shown in Fig. 6. It was noticed that all hydrogel samples showed an interconnected porous structure with a pore size in the range of 100-400 μm. With the increase of CQCOOH photoinitiator concentration from 0.1-0.5 mol %, the pore size and pores distribution of hydrogels decreased accordingly, and surface structure became very tight and uniform, which might be caused by the higher crosslinking density of hydrogels due to increasing free radicals of specimens. This speculation has been further verified by the more careful observation of high irradiated hydrogels. It was found that the pores at hydrogel surfaced decreased gradually with prolonging the irradiation time, due to crosslinking degree has been improved by irradiation time. These results were consistent with the previous results, which claimed that the porosity of scaffolds was reduced clearly with an increase of the crosslinking degree of photopolymerized injectable chitosan-hyaluronic acid hydrogels [19, 20].

Fig. 7

3.6. Effect of CQCOOH photoinitiator concentration on mechanical properties of pullulan-HEMA hydrogels

The *in-situ* photo-rheology was used to monitor and evaluate the mechanical properties of formed hydrogels with different photoinitiator concentration irradiated for 60 seconds, for each different sample. This measurement records the shear storage modulus behavior as a function of oscillation frequency sweep ranged from 0.1-10 Hz after the samples have been illuminated with

a λ~ 465 nm for 60 seconds. The polymer solutions are transferred and crosslinked between two parallel plates, which was built into the as-prepared gels a certain amount of stress. Figure 7 shows the typical data obtained for six CQCOOH concentrations hydrogels (0.1, 0.25, 0.5, 0.75, 1.0 and 1.25 mol %). All hydrogels displayed an increase in storage modulus as a function of CQCOOH concentrations, where the hydrogels with high CQCOOH concentration reached to the leveling off state at (*ca.* 1 Hz) faster than those with low CQCOOH concentration. These data emphasize the kinetic advantages of using a higher CQCOOH concentration in hydrogels. The data elucidate that the shear storage modulus of pullulan-HEMA hydrogels increased progressively from *ca.* 0.5-8.0 kPa, when CQCOOH photoinitiator concentration was raised from 0.1-1.25 mol %. This significant growth of the shear storage modulus with CQCOOH could be attributed to a chain size expansion and further network formation. Furthermore, it was found that the storage modulus increases faster and exceeds the loss modulus of all samples. This increase is characteristics of the alteration of a Newtonian-viscous fluid into an elastic-solid state owing to network formation. Thus, the storage modulus continues to increase due to the gel aging, while the loss modulus was kept invariant and less than storage modulus, showing the dominant elasticity of the system and existence a permanent 3D-network of the hydrogel. It is also of importance to notice that, higher DC% (Fig. 4) does not always result in a higher storage modulus (Fig. 7), because molecular and network structural parameters play major roles in the final physical properties of the mixtures of formed hydrogels, this explanation was also observed by Emami and Soderholm [21]. Similarly, mechanical results are consistent with those of Duchi *et al.* [22], who revealed that the shear storage modulus of gelatin-methacryloyl/ hyaluronic acid methacrylate hydrogels had increased from 0.5-70 kPa when LAP photoinitiator concentration was increased from 0.005-0.1% [22], although the lowest storage modulus hydrogels could achieve the highest shear storage modulus in case prolonging the irradiation time of the photo-polymerization system.

Fig. 8

3.7. Effect of irradiation time on mechanical properties of pullulan-HEMA hydrogels

During the frequency sweep measurements, the shear storage modulus for each different samples have been excelled their loss modulus which indicate that the samples reached to the elastic-solid state (*i.e.* hydrogels). Figure 8 exhibits the prolongation of the irradiation time (10, 30, 60 and 120 seconds) which has influenced adequately on the final storage modulus of formed hydrogels

with different frequency. However, all samples reached to the plateau quickly indicating the polymerization occurred completely even with the shortest irradiation time (10 seconds). Furthermore, prolonging the irradiation time could be considered for enhancing the overall of hydrogel mechanical properties [23]. The results depicted that the storage modulus sharply increased from 1-9 kPa that is probably due to prolonging the irradiation time from 10-120 seconds. These contributions are reliable with those discussed by Duchi *et al.* [22], who suggested that storage modulus of hydrogels increased sharply from 10-70 kPa due to prolonging the irradiation time from 1-10 seconds using LAP photoinitiator. A clear disparity was observed in the final storage modulus in Fig. 8 that is probably owing to the formation of new chemical links related to crosslinking degree and the chains rearrangement improved by the applied shear stress onto the hydrogels. Also, the fast increment in storage modulus is featured by gel transition [22, 23]. Meanwhile, the observed gelation time when storage modulus exceeds loss modulus is found of a paramount for the understanding the hydrogel network formation and morphology of materials.

Fig. 9

3.8. Cytotoxicity test

Toxicity test is regarded as an effective aspect for biomaterials. Ideal biomaterials should not release any toxic substances or produces opposite reactions. Figure 9 shows the cell viability of tested photoinitiating system ingredients (*e.g.* CQCOOH photoinitiator, folic acid coinitiator, and DPITFB accelerator) and crosslinked pullulan-HEMA hydrogels with two different irradiation time for 10 and 60 seconds. The OD of samples obtained from the MTT assay of cells were cultured with the extraction media from various types of specimens. As seen, both CQCOOH photoinitiator and folic acid coinitiator offered non-toxic effect even with a high concentration in comparable media. Unlike, the DPIFFB accelerator exhibited a clear reduction in the percentage of cell viability even with the small cultured cells account, referring that in it was toxic to the cells. For crosslinked hydrogels, the longtime irradiated hydrogels for 60 seconds showed non-toxic effect, compared to those irradiated for 10 seconds which showed also a good viability *ca.* 85-90%. This observation is probably owing to the short time irradiated hydrogels might result in releasing of unconjugated HEMA molecules and unreacted DPITFB specimens which possess a relative toxicity toward the viable cells [24]. It would conclude that the photocrosslinked hydrogels with a high crosslinking degree through high photoinitiator concentration or long

irradiation time was thought non-toxic effect to SW1353, which supports the idea that this photoinitiating system is suitable for bone regeneration and dental applications.

4. Conclusions

Pullulan-HEMA hydrogels were successfully prepared and crosslinked by photopolymerization method using three component-photoinitiating systems based on CQCOOH-folic acid-DPITFB under visible light. The crosslinking performances of hydrogels were described by the degree of conversion by FTIR analysis and crosslinking density using swelling calculations, as a function of variation of both photoinitiator concentration and irradiation time. The final double bond conversion of hydrogel could reach to 90% with CQCOOH concentration of 0.25 mol%, and then it is deteriorated due to a primary premature radical's termination reaction with the high CQCOOH concentration. Also, it could reach to 98% with hydrogel irradiated for 60 seconds. Similarly, the hydrogel network characterization by crosslinking density showed the same trend with photoinitiator concentration and the irradiation time. Moreover, the porosity of hydrogel surface was decreased significantly with an increase of photoinitiator concentration and prolonging the irradiation time, due to increasing of crosslinking density. The storage modulus of hydrogels was increased progressively from 0.5- 8 kPa and 0.5-10 kPa, when photoinitiator concentration was increased and the irradiation time was prolonged, respectively. The indirect cytotoxicity test showed that photoinitiating system ingredients (*e.g.,* CQCOOH and folic acid) did not affect cell viability even with using a high concentration. Conversely, DPITFB showed a toxic effect on cell viability with using a low concentration greater than 0.1%. The study outcomes will thus guide future efforts to modify the system for avoiding toxicity of iodonium salt for the purpose enhancing the cell viability of biomaterials, which is one of the foremost unsolved challenges that deter progress in tissue engineering. Nevertheless, this novel photoinitiating system has the potential to be used as a proper photopolymerization system for scaffolds engineering and preparation of dental materials.

Conflicts of interest

The authors report no financial or nonfinancial conflict of interest.

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