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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 10 s 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 60 s. The current photoinitiating system is a proper system for *in-situ* crosslinking the activated-light biomaterials for bone regeneration, dental, or tissue engineering applications.

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 min), 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.

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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 s, very strong photobleaching, 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 14,000*, 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 s.

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 min 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 min 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 (5 mm *thick.* and 25 mm *diam.*) of hydrogel disk. The hydrogel was formed after <1 min 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].

$$ESR\% = (W_s - W_d)/W_d \times 100. \quad (1)$$

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].

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

where, $_v$ is the specific volume of dry polymer (0.614 cm^3 g^{-1} at 20 °C 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