

Photopolymerized Injectable Water-soluble Maleilated Chitosan/ Poly(ethylene glycol) Diacrylate Hydrogels as Potential Tissue Engineering Scaffolds

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Photocrosslinkable water-soluble maleilated chitosan was synthesized by mixing high molecular weight of chitosan with maleic anhydride under mild and heterogeneous reaction conditions using dimethyl sulfoxide as a solvent. And then, maleilated chitosan (MCS)/ poly(ethylene glycol) diacrylate (PEGDA) hydrogels were prepared under UV radiation. Series of properties of the hydrogels including rheological property, swelling behavior, morphology and mechanical test were investigated. The results showed that, the MCS/PEGDA hydrogels had faster gel-forming rate, higher compressive strength than MCS hydrogel. The swelling behavior and mechanical properties of the hydrogels were also tunable via the control of weight ratio of MCS to PEGDA. The indirect cytotoxicity assessments of the hydrogels were studied. The result showed the photocrosslinked hydrogels was compatible to L929 cells at low dosages. Cell culture assay also demonstrated that the hydrogels were good in promoting the L929 cells attachment, showing their potential as tissue engineering scaffolds.

Keywords: Hydrogel, Chitosan, Water-solubility, Photopolymerization, Tissue engineering

1. Introduction

Photopolymerized injectable hydrogels are extensively investigated for various tissue engineering applications [1,2], primarily due to their unique merits such as direct application to the targets in a minimally invasive manner, spatially-controlled fast gel transition at physiological condition [3], and more importantly, local delivery of growth factors or cells to promote tissue repair rapidly by mimicking natural tissues environments [4]. Several synthetic and natural polymers, such as poly(ethylene glycol) [5], hyaluronic acid [6], alginate [7] and chitosan, have been explored to synthesize photocrosslinked injectable hydrogels. Among them, chitosan is one of the most promising biomacromolecules to prepare the hydrogels for tissue engineered

scaffolds, attributed to its excellent biological properties such as biodegradability, biocompatibility and wound-healing activity [8]. Further, chitosan has been shown to mimic the glycosaminoglycan rich extracellular matrix of tissue such as articular cartilage [9].

Photocrosslinked chitosan hydrogel has been reported by many researchers [3,10]. Considering hydrogels formation at physiological condition as crucial requirement for tissue scaffolds, chitosan often is modified by water-soluble units so as to change intrinsic acid-soluble property of chitosan and then grafted with photocrosslinkable groups along its chemical chains. Methacrylated *O*-carboxymethyl chitosan [11], Methacrylated *N*-succinyl chitosan [12], methacrylated glycol chitosan [13], azidobenzaldehyde modified

carboxymethyl chitosan [14], and azidobenzoic acid-modified lactose chitosan [15] was synthesized. However, these synthesis protocols normally involve multistep chemical modification, which could lead to relatively low yields of final products. Besides, azo-derivitized chitosan hydrogels was found to have high storage moduli or pore sizes that are not ideal for neurite out growth [1]. A simple one-step modification of chitosan, photocrosslinkable water-soluble maleic chitosan (MCS), was also prepared [16]. However, formic acid was used as a solvent in this reaction condition, which could result in degradation of chitosan molecular chain to make final hydrogels relatively weaker. Additionally, the trace toxic acid solvent in hydrogel products is harmful when it is applied to human tissue.

Herein, a photocrosslinkable water-soluble maleic chitosan with high molecular weight chitosan as a raw material was synthesized under mild and heterogeneous reaction conditions using dimethyl sulfoxine as a solvent. Maleic chitosan was as a precursor to blend with a photoinitiator and water to create injectable hydrogels under UV irradiation. Here, poly(ethylene glycol) diacrylate (PEGDA) was selected as a crosslinking agent to reinforce chitosan hydrogel network, in addition to properties of PEG hydrogels such as relative inertness, biocompatibility and promotion of cell growth [17]. The MCS/PEGDA hydrogels were prepared and series of properties including rheological property, swelling behavior, morphology and mechanical test were investigated. The indirect cytotoxicity, cell attachment, and cell proliferation were investigated as well.

2. Experimental

2.1. Materials

Chitosan (CS, viscosity = 80 mPa·s, degree of deacetylation = 84.5%) was purchased from Jinhu Crust Product Co., Ltd., China. Maleic anhydride (MA) was purchased by Sinopharm Chemical Reagent Co, Ltd. Polyethylene glycol diacrylate 600 (PEGDA600, Sartomer Company, Inc, USA) was used as a crosslinking agent without further purification. Cytocompatible UV photoinitiator Darocur 2959 (D-2959, 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone) was obtained

from Ciba-Geigy Chemical Co. (Tom River, NJ). Mouse fibroblasts (L929) were obtained from Wuhan Beinglay Biological Technology Co., Ltd., China. Other reagents were all A.R. grade.

2.2. Synthesis of water-soluble maleilated chitosan (MCS)

A modified chitosan carrying vinyl carboxylic acid groups was designed and synthesized for further photopolymerization. Briefly, 1.0 g of chitosan was suspended in 100 mL of dimethyl sulfoxide (DMSO). Maleic anhydride (3.5 g) was dissolved in 10 mL of DMSO and then added into the flask dropwise for 20 min. The reaction solution was allowed to sit for 24 h at 60 °C. After that, saturated NaHCO₃ solution was added to the reaction mixture to adjust the pH to 8-9 in order to convert the carboxylic acid to its sodium salt. The mixture was precipitated by acetone and then dialyzed (membrane molecular weight cut-off 12000 g·mol⁻¹) against water for 2 days and lyophilized to obtain pure MCS. ¹H NMR spectrum was recorded on a Bruker AV 400 NMR instrument.

2.3. Preparation of photopolymerized hydrogel

The hydrogel was achieved by mixing a 6.0 wt% MCS aqueous solution with various amounts of PEGDA containing 0.05 wt% D-2959 (relative to amount of blend solutions) at MCS/PEGDA weight ratios of 2:1, 1:1 and 1:2, respectively. The solution was transferred into a disk-shaped mold consisting of two glass microslides separated by a spacer, then irradiated with an Omnicure Series 1000 UV light source (60 mW/cm², Exfo, Canada) for 30 min at ambient temperature.

2.4. Rheological measurements

In situ dynamic photorheology [18] was used to measure the elastic and viscous moduli during photopolymerization. A Haake Mars Rheometers (Thermo Fisher Scientific Inc.) equipped with a UV curing attachment and 20 mm parallel plate geometry was used to characterize the photocrosslinking kinetics. The upper plate was made of an optically transparent quartz acting as filter for UV light with a cutoff of 320-480 nm. The gap setting was fixed as 1.0 mm. UV light intensity (60 mW/cm²) were used for the crosslinking

reaction of the precursor. Time-sweep oscillatory tests were performed at 25 °C at strain amplitude of 1.0% and a 6.28 rad/s, which was within the linear viscoelastic region [19]. The storage and loss modulus values were continuously recorded by Haake RheoWin measuring and evaluation software.

2.5. Swelling characterization

Lyophilized photopolymerized MCS/PEGDA hydrogels were submerged in phosphate buffered saline (PBS) at 37 °C. Swollen gels removed from the solution at regular intervals were dried superficially with filter paper, weighted. The measurements were continued until a constant weight was reached for each sample. The degree of swelling, Q , is expressed as the amount of absorbed water per gram of dry polymer during a regular time interval. $Q = (W_s - W_0)/W_0$, where, W_s and W_0 are the weights of the samples in the swollen and dry state, respectively.

2.6. Sol determination

The lyophilized MCS/PEGDA hydrogels (dry mass recorded as m_0) were swollen three times in purified water at 37 °C, with the water replaced every 45 min. The gels were again frozen, lyophilized and the final mass recorded as m_1 . The sol content was calculated as: $\text{sol} = (m_0 - m_1)/m_0$.

2.7. Mechanical test

Unconfined compression testing of the MCS/PEGDA hydrogels was carried out on an Instron 5848 microtester (Instron, Norwood, MA, USA) with 10 kN load cell at a compression rate of 0.5 mm/min. The fracture stress and strain were determined with the failure point of the stress-strain curve. Three samples of each type of hydrogels were examined in this experiment.

2.8. The morphology of hydrogels

To visually examine the surface morphology of hydrogels, a JEOL Model JSM-6510 scanning electron microscope (SEM) was used to analyze the pore structure. The freeze-dried samples were loaded on the surface of an aluminum SEM specimen holder and sputter coated with gold before observation. The accelerating voltage was 20 kV.

2.9. Cytotoxicity assays

Extracts were prepared from MCS/PEGDA hydrogels by adding fragments of the sterile samples to the culture medium at a concentration of 1.25 cm²/mL and incubating at 37 °C for 24 h without shaking. After this period, the medium was obtained and the MCS/PEGDA hydrogels were removed. This procedure aimed to assess potential deleterious effects of substances released by MCS/PEGDA hydrogels into the culture medium.

According to ISO-10993, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to determine the extracts toxicity. Initially, 200 µL of L929 cell suspension at density of 10⁵ cells per well were seeded in wells of 96-well plate and cultured for 24 h at 37 °C. After this period, the culture medium was removed and replaced with 100 µL of as-prepared extraction medium. After 24 h at 37 °C, the extracts were removed and 10 µL of MTT solution was added to each well. After 4 h incubation at 37 °C, 150 µL of dimethyl sulfoxide was added to dissolve the formazan crystals. The dissolved solution was swirled homogeneously for about 10 min by the shaker. The optical density of the formazan solution was detected by an ELISA reader (Multiscan MK3, Labsystem Co. Finland) at 570 nm.

For reference purposes, cells were seeded to a fresh culture medium (negative control) under the same seeding conditions, respectively.

Results are depicted as mean ± standard deviation. Significance between the mean values was calculated using ANOVA one-way analysis (Origin 7.0 SRO, Northampton, MA, USA). Probability values $p < 0.05$ were considered significant ($n = 6$).

2.10. Cell culture and adhesion

To assess the behavior of cells on MCS/PEGDA hydrogels, a cell attachment/migration study was used. The lyophilized MCS/PEGDA (1:2) were fixed and then were sterilized and extensively washed three times with sterile PBS prior to transfer to individual 24-well tissue culture. Aliquots (1 mL) of mouse fibroblasts (L929) suspension with 1.0 × 10⁶ cell/mL were seeded on the sample membranes. After 24 h of culture, cellular constructs were harvested, rinsed twice with PBS to remove non-adherent cells

and subsequently fixed with 2.5% glutaraldehyde for 2 h. After that, the samples were lyophilized and sputtered with gold for observation of cell morphology on the surface of the scaffolds by SEM.

3. Results and Discussion

3.1. Synthesis of water-soluble MCS

Chitosan are insoluble in water because of intra- and intermolecular hydrogen bonding [20], which limits its application in tissue engineering. However by disrupting these hydrogen bonds, the water-soluble MCS was prepared by a simple one step chemical reaction between maleic anhydride and chitosan in DMSO. Here, viscosity-average molecular weight of chitosan (80 mPa·s) determined by viscometer was 1.02×10^6 . And polymer backbone degradation was not obvious under heterogeneous mild reaction conditions due to no acid involved because acid condition resulted in significant polymer backbone degradation, and then lead to strength reduction of hydrogels formed. Furthermore, MCS with maleyl group can easily processed into a crosslinked hydrogel via radical polymerization across carbon-carbon double bonds using D-2959 initiation under UV irradiation.

The ^1H NMR spectra of water-soluble MCS and CS are shown in Fig. 1. The incorporation of double bonds into the chitosan backbone was confirmed by ^1H NMR, as indicated by the appearance of proton signals at 5.6 ppm and 6.2-6.5 ppm due to protons on the vinyl carbon. The degree of substitute (DS) of maleic groups onto the chitosan backbone was calculated from the relative integrations of the vinyl protons at 5.7 ppm with respect to the methyl protons of $-\text{NHCOCH}_3$ at 1.7 ppm: $\text{DS} = 3 \times 0.155 \times I_{5.7} / I_{1.7}$. By calculation, DS was 1.67 for MCS. Other peaks were attributed as follows. 4.3 ppm (H-1 of GlcNAc), 3.3-3.6 ppm (H-3,4,5,6 of N-alkyl group, GlcN and GlcNAc).

3.2. Rheological measurements

Rheological time sweep tests were performed using a UV light attachment at 25 °C for in situ gelation to investigate the effect of different MCS/PEGDA ratio on the crosslinking kinetics and rheological properties of the hydrogels, as shown in Fig. 2. As can be seen, a typical

evolution of gelation is observed, revealing a polymerization process identified by three different phases: viscous liquid phase ($G' < G''$), onset of gelation ($G' = G''$) and solid-like gel phase ($G' > G''$) [21]. The kinetics of the gelation process was significantly affected by varying the MCS/PEGDA ratio. For pure MCS hydrogel, onset of gelation occurred at 276 s and gelation finished within 900 s. However, gelation point of MCS hydrogels reduced from 120 to 52 s with PEGDA/MCS weight ratio increased from 1:2 to 2:1. These results were associated with higher monomer concentration caused by increasing fraction of PEGDA. In free radical polymerization, the polymerization rate is proportional to monomer concentration, so higher monomer concentration contributes more rapid polymerization rate, which favors shorting gelation time.

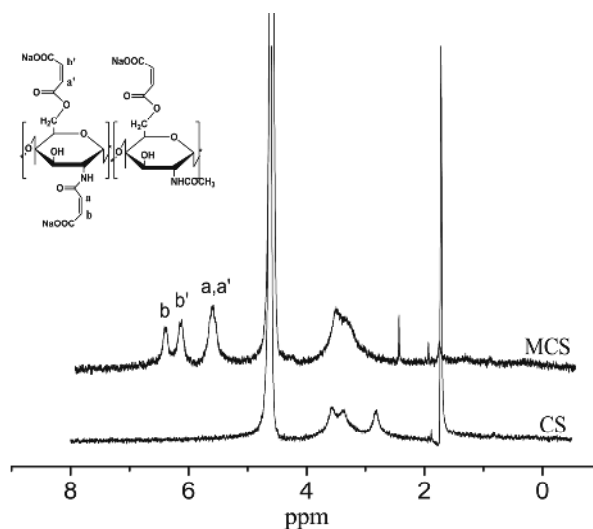


Fig. 1. ^1H NMR spectra of MCS and CS with $\text{CD}_3\text{COOD}/\text{D}_2\text{O}$ as solvent.

3.3. Swelling characterization

Figure 3 shows the water content of MCS/PEGDA hydrogels measured at various time intervals. It could be seen that, all MCS/PEGDA hydrogels generally showed a high swelling rate during the initial 10 min, leveling off thereafter and finally reaching equilibrium within 200 min.

As shown in Fig. 3, as relative amount of PEGDA increased, the MCS/PEGDA hydrogels exhibited faster swelling rate and less equilibrium swelling ratios. The swelling

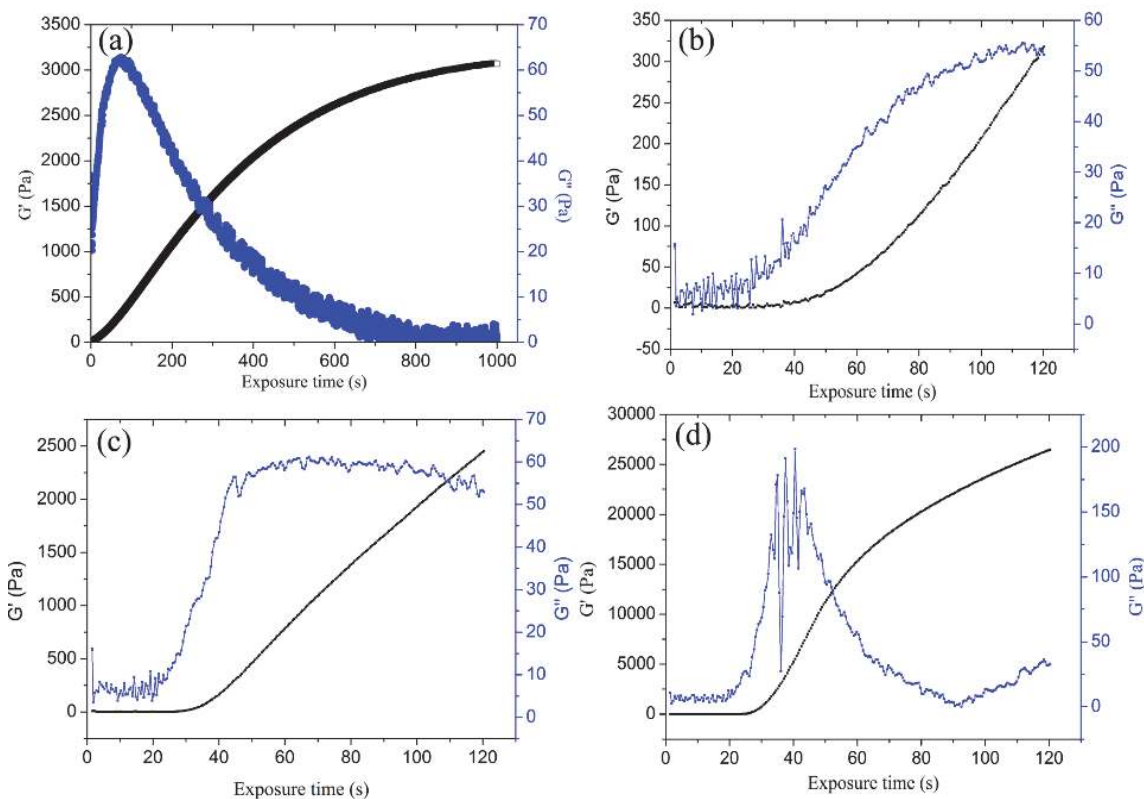


Fig. 2. In situ polymerization towards MCS/PEGDA hydrogels with variation in MCS to PEGDA ratio. (a) MCS; (b)2:1; (c)1:1; (d)1:2.

ratio of the hydrogels (MCS/PEGDA=1:1 or 1:2) could be above 90% of equilibrium swelling ratio within 7 min, while that of the hydrogels (MCS/PEGDA=2:1) could just get to 75.3% with the same time. And the hydrogels (MCS/PEGDA=1:2) had lowest equilibrium swelling ratio of 6.3, lower than that of the hydrogels (MCS/PEGDA=2:1). This behavior could be due to increase in crosslinking density of the MCS/PEGDA network from enhancement of molecular entanglement between MCS and PEGDA with PEGDA content increased, which led to form a close network structure, leaving less free water molecules to diffuse into the network so as to decrease of its water absorbing ability.

As also observed, the MCS/PEGDA hydrogels could absorbed over 6 times their dry weight in water within 12 min, which showed the MCS/PEGDA hydrogels had fast swelling kinetics and relatively high water retention capability, probably due to their hydrophilic nature, which favored their applications in rapid hemostasis. Not all the hybrid hydrogels reported in the papers show such fast and relatively high levels of swelling.

Han [10] prepared methacrylated chitosan/PNIPAAm hybrid hydrogels with highest equilibrium swelling ratio of 1.6 and shortest equilibrium time of 500 min. Chen [22] prepared thiolated chitosan/PEGDA/glycerophosphate hydrogels having highest swelling ratio below 1.3 and equilibrium time exceeding 5 days.

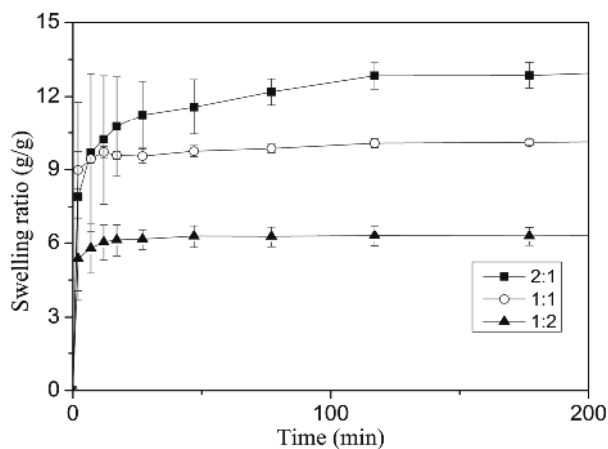


Fig. 3. Swelling kinetics of MCS/PEGDA hydrogels with different MCS/PEGDA ratio.

3.4. Sol determination

An important parameter in the manufacture of hydrogel scaffolds for tissue engineering applications is the sol content of the network formed. A relatively low sol content means efficient crosslinking. After photopolymerization, not all of the prepolymers are reacted into the hydrogel network. It was observed that MCS/PEGDA hydrogels have low sol contents (0.060-0.070) (Table 1), lower than neat MCS hydrogel of 0.145 (no shown). Hydrogen bonding between MCS and PEGDA might account for the lower sol content. In addition, chain flexibility may be another factor to affect sol content. MCS has lower chain flexibility than PEGDA [23], which may lead to higher sol content of MCS hydrogel. Similar results was also reported in the literature [24]. However, there was no significant difference between sol contents of hydrogels with different MCS/PEGDA weight ratio, which showed that there was little influence of MCS/PEGDA weight ratio on hydrogel sol contents at a given photocrosslinking condition.

Table 1. Formulations and characteristics of MCS/PEGDA hydrogels.

No	Weight ratio (g/g)	Water uptake (g/g)	Sol fraction (g/g)	Compressive strength (MPa)
1	2:1	12.85 ± 0.54	0.070 ± 0.020	0.062 ± 0.009
2	1:1	10.07 ± 0.18	0.064 ± 0.011	0.051 ± 0.014
3	1:2	6.30 ± 0.41	0.060 ± 0.016	0.322 ± 0.069

3.5. Mechanical test

The mechanical properties of MCS/PEGDA hydrogels are listed in Table 1. It could be seen that, the MCS/PEGDA hydrogels have low compressive strength of 0.062±0.009 MPa at MCS/PEGDA weight ratio higher than 1:1, while compressive strength of MCS/PEGDA hydrogels gets to 0.322±0.069 MPa when MCS/PEGDA=1:2, close to native tissue such as bovine cartilage tissue (0.35±0.1 MPa) [25]. This behavior is a result of increase in crosslinking density of the MCS/PEGDA network with PEGDA content increased. Here, the MCS/PEGDA system has favorable physical properties when it is used as cartilage repair materials. The MCS/PEGDA system exists as a liquid at room temperature, which allow it to be injected into an injured joint and infiltrate deep into matrix cracks and fracture

edges, and then transitioned to a gel under UV radiation to offer relatively sufficient mechanical strength to damaged cartilage and helped to restore native cartilage.

3.6. The morphology of hydrogels

Microstructures of the networks cross-section investigated by scanning electron microscopy are presented in Fig. 4. It could be seen that, freeze-dried MCS/PEGDA hydrogel exhibits seaweed-like porous structure at higher MCS/PEGDA weight ratio with distribution of porosity range from 12 to 257 μm, while MCS/PEGDA hydrogel with low weight ratio (1:2) shows relatively homogeneous and dense pores ranging from 36 to 187 μm. With PEGDA content increased, the equilibrium water content of MCS/PEGDA hydrogels decreased, which led to smaller pore size in the hydrogels obtained from lyophilization. In addition, crosslinking density [10], molecular dimensions and hydrophilicity of the polymer have influence on pore morphology. Figure 4c also shows the co-continuous or interconnect open porous structure, which indicated PEGDA as a scaffold responsible for the framework of the final product, favoring to the cell adhesion and proliferation [26]. Our interior morphological data are also consistent with compressive moduli data discussed above.

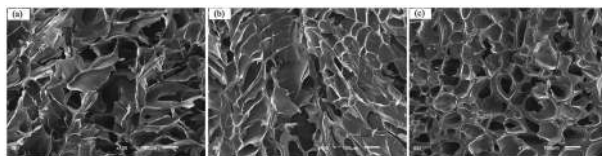


Fig. 4. SEM images of MCS/PEGDA hydrogels at different weight ratios. (a) 2:1; (b) 1:1; (c) 1:2.

3.7. Cytotoxicity assays

An ideal tissue engineering scaffold should not release toxic products or produce adverse reactions, which could be evaluated through in vitro cytotoxic tests. In the evaluation, mouse fibroblast cells (L929) were used as reference. Figure 5 shows the absorbance obtained from an MTT assay of L929 cells which were cultured with the extraction medium from various samples in comparison with control. It could be seen that, statistically significant differences ($p < 0.05$) were observed in the cell activity of L929 culture for 24 h in the

presence of photocrosslinked MCS/PEGDA hydrogels when diluted extract concentration above 50% was used. Although statistically significant differences ($p < 0.05$) were observed in the cell activity in comparison with control when 100% extract was used, the viability of the cell still reached 79% of that of the negative control. This indicated that photocrosslinked MCS/PEGDA hydrogels were less toxic to L929 cells. The obtained results clearly suggested that photocrosslinked MCS/PEGDA hydrogels had relatively biocompatibility and were good candidates to be used as tissue engineering scaffolds.

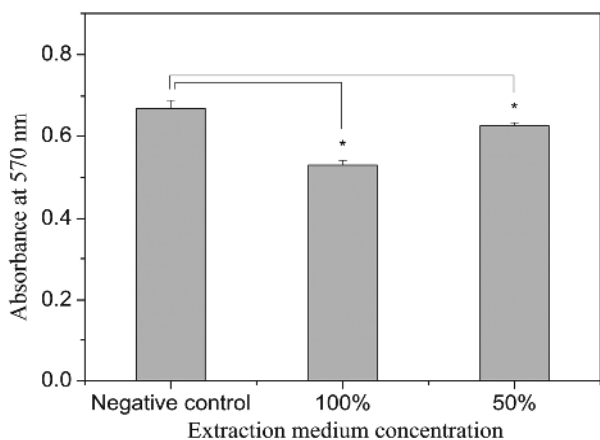


Fig. 5. Cytotoxicity test of photocrosslinked MCS/PEGDA hydrogels (MCS/PEGDA=1:2) with negative controls ($p < 0.05$) * $p < 0.05$ when compared to the negative control of indirect cytotoxicity.

3.8. Cell adhesion and morphology

For a material possibly used in biomedical application, its biological compatibility is also evaluated by attachment of cells on the surface of it. Figure 6 shows SEM image of L929 cells that were cultured on photocrosslinked MCS/PEGDA hydrogels scaffolds at 37 °C for 24 h. As observed, L929 cells appeared to adhere well and exhibited a normal morphology on the surface, which was probably due to cell adhesion site existed on the hydrogel surfaces via the attachment of media proteins cells synthesized to the hydrogel surfaces for cell attachment. The evidence of cell-to-cell interaction is indicative of non-cytotoxic response of the cells to the substrate, suggesting that the hydrogel could have good biocompatibility.

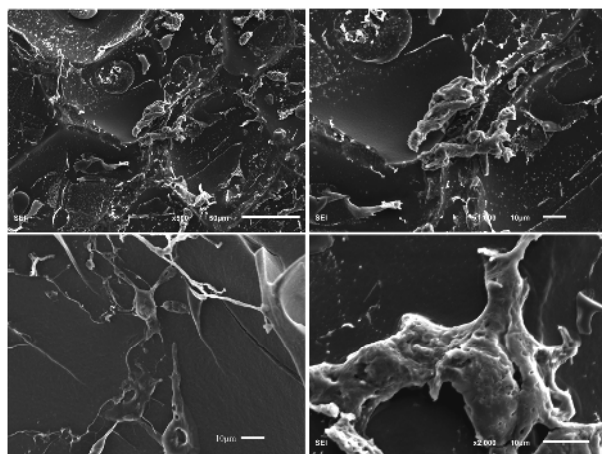


Fig. 6. SEM images of L929 cells seeded on MCS/PEGDA hydrogel (MCS/PEGDA=1:2) after 24 h culture.

4. Conclusion

Photocrosslinkable water-soluble maleilated chitosan was synthesized and therefore MCS/PEGDA hydrogels were prepared by photopolymerization technique. The MCS/PEGDA hydrogels had faster gel-forming rate, higher compressive strength than MCS hydrogel. The swelling behavior and mechanical properties of the hydrogels were also tunable via the control of weight ratio of MCS to PEGDA. The result of indirect cytotoxicity assessments showed the hydrogels as compatible to L929 cells at low dosages. Cell culture assay also demonstrated that the hydrogels were good in promoting the L929 cells attachment, showing their potential as tissue engineering scaffolds.

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