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Assessment of Centella Asiatica Extract Containing Dual-crosslinked Gel-MA/Pec Hydrogels as Wound Dressing

Betul Albayrak[1] Melisa Gelal[1] Demet Akalgan[2] Burcin Izbudak[3] Bengi Ozkahraman[4] Ayca Bal-Ozturk[1,3,5]

[1]Istinye University, Department of Analytical Chemistry, Istanbul, Turkey
[2]Istinye University, Department of Pharmacognosy, Istanbul, Turkey
[3]Istinye University, Department of Stem Cell and Tissue Engineering, Istanbul, Turkey
[4]Hitit University, Department of Polymer Engineering, Corum, Turkey
[5]Istinye University, 3D Bioprinting Design & Prototyping R&D Center, Istanbul, Turkey

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***Correspondence to**: Ayca Bal-Ozturk, Istinye University, Department of Analytical Chemistry, 34010, Istanbul, Turkey.*
E-Mail: aozturk@istinye.edu.tr, aycabal@ gmail.com

ABSTRACT

In the present work, 3D-printed wound dressings containing different amounts of Centella Asiatica (CA) extract were synthesized via dual-crosslinking method. Methacrylic anhydride modified gelatin (Gel-MA) and pectin (Pec) were chosen as the base material for dressings. A dual crosslinked network was formed with Gel-MA photo-crosslinking using Irgacure 2959 and Pectin (Pec) psychical-crosslinking using Ca^{++} ions. Meanwhile, the developed dual-crosslinked hydrogel dressings were characterized by Fourier transform infrared spectroscopy, scanning electron microscopy, and mechanical, swelling, *in vitro* degradation as well as ex vivo bioadhesion tests. As the amount of CA in the dressing hydrogels increased, the degradation rates increased. In addition, the prepared dressings provide good bioadhesion on the chicken skin. Moreover, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay revealed cell viability of Gel-MA/Pec hydrogel dressings containing CA extract. The results presented the dressings as a promising biomaterial to be used for wound healing purposes, which should be further investigated in future.

Keywords:
Centella Asiatica extract; 3D-printing; Wound dressing; Dual crosslinking; Pectin.

INTRODUCTION

Wound infections, diabetes, weak immune system, severe obesity, malnutrition, and long surgery times are the main factors leading to acute or chronic wounds that put a physical, mental, social, and economic burden on patients [1]. Recently, there is a lot of scientific work being done to explore some innovative wound dressings that are capable of wound healing process [2-4]. These materials have many advantages compared to conventional materials (gas, rosette, etc.), such as absorbing exudates, and providing flexibility, softness, and air/water permeability [5]. Beside their hydrophilic nature, dressings have accommodated bioactive inorganic/organic substances; these bioactive materials could be released into the wound zone [6]. These significant properties provide a unique advantage in clinical and biomaterial applications [7-9]. The 3D-printing technology proceeds gaining importance in the area of biomedical engineering such as regeneration medicine and tissue engineering which has the capability to supply customised innovative solutions in the biomaterial market [10]. It is possible to rapidly prototype or customized a final product design by 3D-printing technique with a digitally controlled layer-by-layer deposition methods via computer-aided design (CAD) [11].

Centella asiatica (CA), is known locally as pegaga nyonya, is extensively used in traditional medicine because of its antioxidant [12], antifungal [13], anti-inflammatory [14], antidiabetic [15], as well as antibacterial [16] properties. Also, CA can be used in the formulation of wound dressings to improve the healing property by encouraging collagen synthesis and fibroblast proliferation [17, 18]. Thus, the interest in using CA extracts for wound dressing applications is progressing, and several biomaterials have been investigated [19].

Polysaccharides have been widely used as versatile basic materials for various applications in biomedical and biotechnological areas due to their non-toxic and biocompatible behaviours [20-21]. However, the poor mechanical properties limit their use as biomaterials [22]. In the meantime, a few modified natural polymers, such as alginate [23], chitosan [24], gelatin [25], gellan gum [26], and kappa carrageenan [27] were used to improve the mechanical properties via photo and/or chemical crosslinking. Conventional Gel-MA is a commonly used biomaterial that was formed by conjugation of methacrylate units to the amine-containing side units of Gel backbone [28]. Gel-MA could be chemically-crosslinked with the presence of a photoinitiator via ultraviolet (UV) light [29]. Pec is an anionic polysaccharide that is extracted from plant cell walls, and it is generally used as a biomaterial due to its distinctive characters such as easy availability, biocompatibility, and adhesiveness [30-31]. Pec has numerous carboxyl (-COOH) and hydroxyl (-OH) groups on its backbone, which easily form a physical/ionic crosslink with Ca^{++} (positively charge) [32].

In this study, Gel-MA/Pec based wound dressings containing different amounts of CA extract were fabricated by using a 3D-printer. 3D-printed wound dressings had a dual crosslinked network that formed psychically crosslinking with Ca^{++} ions and photo crosslinking under UV light with Irgacure 2959. The chemical structures of 3D printed wound dressings were evaluated by Fourier transform infrared spectroscopy (FTIR). To assess the morphology of the dressings, scanning electron microscopy (SEM) was used. The mechanical and ex vivo bioadhesion behaviors of Gel-MA/Pec dressings were evaluated using elongation at break, compression strength, and bioadhesive force results. The dressings materials were also analyzed *in vitro* cytotoxicity test against NIH/3T3 cells. These results provide that CA containing Gel-MA/Pec 3D-printed materials are encouraging candidates for use in wound dressing operations.

MATERIAL AND METHODS

Chemicals

Pec, phosphate buffer saline (PBS) tablet, Irgacure 2959, 2,2-diphenyl-1-picrylhydtazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium and Ham's F-12 (DMEM/F-12), penicillin-streptomycin (P-S) and fetal bovine serum (FBS) were supplied from Capricorn Scientific GmbH (Germany), Thermo Fisher Scientific, (USA) and PAN-Biotech (Germany), respectively. $CaCl_2$ was supplied from Merck. CA was obtained from Bayer. Gel-MA was obtained as a gift from AdBioInk Biosystem Technology Corp. (Turkey). The chemicals used in experimental work were of analytical degrees.

Preparation procedure of Gel-MA/Pec wound dressing materials

3D-printed Gel-MA/Pec based wound dressings were prepared by a combination of photo-crosslinking and physical-crosslinking methods. Briefly, Pec (40 mg) was dissolved in deionized water (2 mL) containing 0.25% (w/v) Irgacure 2959 under stirring for 1 hour at 80 °C. For CA incorporated wound dressings, the different amounts of CA were added to Pec solutions with continuous stirring for 1 hour at 50 °C. Gel-MA (300 mg) was added to the prepared mixtures and stirred for 1 hour at the same temperature. Subsequently, the pre-polymer solutions were transferred into the barrel systems. Square structures (10 x 10 mm) were printed with Axodual Bioprinter (Axolotl Biosystems) with a speed of 10 mm/seconds at room temperature. Then, the printed structures were crosslinked by first keeping them under UV light (360-480 nm) for 240 seconds (Omnicure S200, Excelitas Technologies) and then immersing them in 1% (w/v) $CaCl_2$ solution for 3 minutes [30]. According to the increasing CA concentrations (0, 10, 20, and 40 mg/mL), the 3D-bioprinted wound dressing hydrogels were referred to as Gel-MA/Pec-CA-0, Gel-MA/Pec-CA-0.5, Gel-MA/Pec-CA-1, and Gel-MA/Pec-CA-2, respectively.

Characterization

Structural analysis of 3D-printed wound dressings was performed by ATR-FTIR (Jasco FT/IR-4600) at 500-4000 cm^{-1} wavelength. The surface morphology of the wound dressing materials was explored by using SEM (Zeiss Sigma 300).

The swelling degree of the hydrogel dressings consisting different amounts of CA was determined [33]. Briefly, the small pieces with an equal weight of wound dressing hydrogels were immersed in PBS (pH 7.4) medium at 37 °C. The samples were taken out at different periods (24 and 48 h), after which the surface water was gently eliminated from the swollen wound dressings with filter paper. Finally, the swollen samples were immediately weighed. The swelling degree of the dressings was quantified from the following equation;

$$Swelling\ \deg ree\ (g/g) = \frac{W_{swollen} - W_{dried}}{W_{dried}} \qquad (1)$$

where $W_{swollen}$ is the swollen sample and W_{dried} is its dry weight. Each sample was measured in a quadruplicate.

The hydrolytic degradation properties of the hydrogel wound dressings were calculated by weighing the dri-

ed dressings before immersion in PBS (pH 7.4) medium at 37 °C. Then, the wound dressings were removed from the degradation medium at different periods (7th, 14th and 21st days) and lyophilized. Afterward, the dried samples were weighed. The degradation rate (%) was determined according to the equation below [34];

$$Degradation\ (\%) = 100 - (\frac{W_{before}}{W_{after}} x100) \quad (2)$$

where W_{before} was the weight of the dried dressings before degradation process, and W_{after} was the weight of the dried dressings after degradation process. Each sample was measured in a quadruplicate.

Mechanical test

Tensile strength and elongation at break measurements of the hydrogel dressings were performed by a texture analyzer (Stable Micro Systems). Prior to testing, the dressings were cut into discs (6 mm width x 100 mm length) using a biopsy punch, and set to the device under static-load conditions. The initial grip separation was fixed at 5 mm. Further, the dressings were stretched at a speed of 10 mm/min [35]. Each sample was measured in a quadruplicate.

Bioadhesion test

The chicken-skin was used to consider the ex vivo bioadhesive futures for the Gel-MA/Pec based dressings by using the TA.XT plus Texture Analyser. 2x2 cm² of chicken-skins were attached to a bioadhesive ring and wetted with PBS (pH 7.4). The dressings were fixed with cyanoacrylate glue to the device. Afterwards, the test was done at 0.5 mm/sec speed and device came intact with the chicken-skin for 3 minutes (0.5 N force was applied). To seperate the hydrogel dressings from the surface of chicken-skin, the device was withdrawn at 0.5 mm/sec speed from the surface of chicken-skin. Bioadhesion force (F_{max}) was recognized as the maximum force enforced to completely detach the hydrogel dressings from the chicken-skin [36]. The test was repeated with three samples.

Cell viability assay

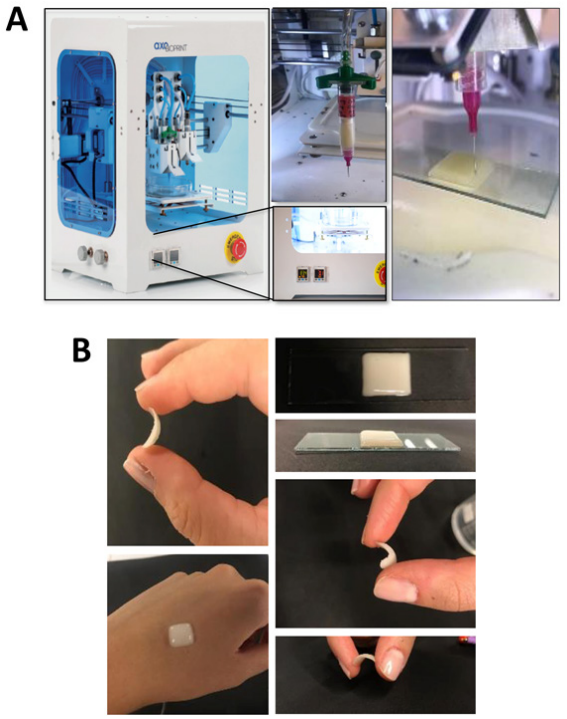
The cytotoxicity of 3D-printed dressings was evaluated by an indirect MTT test [37]. NIH/3T3 cell line was used. DMEM/F-12 containing 10% FBS, 1% L-glutamine and 1% P-S was used as culture medium. First, 2 mL of DMEM F-12 culture medium was added to the disc-shaped hydrogel dressings containing different proportions of CA and incubated for 24 hours at 37 °C in a humidified incubator with 5% CO_2. Then, DMEM/F-12 containing sample extracts was filtered and sterilized with a 0.22 µm syringe filter. NIH/3T3 cells were seeded at 5x10³ cells/well and incubated for 24 hours at 37°C with 5% CO_2. The cells were then replaced with culture media treated with hydrogel dressings and incubated for 24 hours. Cell images were taken with an inverted microscope (Axiovert 25Carl Zeiss). Then, 100 µl of DMEM/F-12 and 10 µl of MTT (5 mg/mL in PBS) solution were added to wells and incubated for 4 hours. Fresh DMEM F-12 was used as a control. Then, DMEM/F-12 consisting 10% MTT was removed and 100 µL of DMSO was added to the wells and incubated at 37°C for 20 minutes. The obtained solution was quantified with a microplate-reader at 570 nm.

RESULTS AND DISCUSSION

Characterization of the hydrogel wound dressings

3D-printed Gel-MA/Pec based wound dressings were prepared with Gel-MA, Pec, and different amounts of CA extract. The 3D-printing process of the dressings and 3D-printed structures were presented in Scheme 1. The structural characterization of the dressings was recorded by ATR-FTIR and the results were presented in Figure 1-A and 1-B. The characteristic bands of Pec were detected at 3342 and 2930 cm^{-1} due to the presence of hydroxyl (–OH) and aliphatic –CH stretching vibrations, respectively [31]. Gel-MA was also related to the C-N-H vibratory bond, and the band within the range of 3200–3400 cm^{-1} revealed the peptide bonds (mainly stretching NH bonding) [38]. The formation of the dressings was verified by the following bands; the wide absorption band at 1630 cm^{-1} was related to C=O stretching and the band at 1542 cm^{-1} was attributed to the stretching vibration of C-N-H [39]. Moreover, the absorption band from the hydroxyl unit (-OH) near 3300 cm^{-1} was observed in the spectra of Gel-MA, which meant the reduced number of hydrogen double bonds because of the reaction of amino units (-NH) in Gel after the reaction with the methlacrylate group [39, 40]. In addition, the second network of Gel-MA/Pec 3D printed wound dressings showed a band at 1727 cm^{-1} due to the interaction of negatively charged COO^- units of Pec with positively charged Ca^{++} ions [41]. Moreover, there was not much change observed in the peak presence of CA extract due to overlap amid bands of Gel-MA and CA [42].

The surface morphologies of Gel-MA/Pec-CA-0 and Gel-MA/Pec-CA-0.5 3D printed wound dressings were investigated with SEM analysis. As shown in Figure 2-A, it could be observed smooth waves on the surface of the Gel-MA/Pec-CA-0. The CA addition to the formulationx did not change the surface area of the dressing's smooth appearance (Figure 2-B). The result demonstrated that the CA dis-



Scheme 1. (A) 3D-printing process of the Gel-MA/Pec-CA hydrogel dressings and (B) 3D-printed structures.

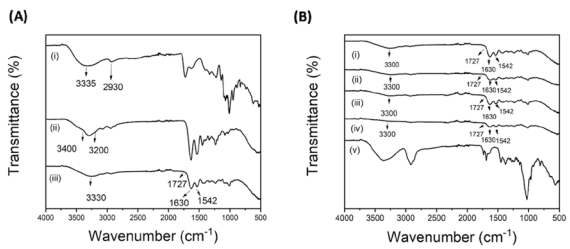


Figure 1. (A) FTIR spectra of the Pec (i), Gel-MA (ii), and (iii) Gel-MA/Pec-CA-0 (B) FTIR spectra of the hydrogel dressings: Gel-MA/Pec-CA-0 (i), Gel-MA/Pec-CA-0.5 (ii), Gel-MA/Pec-CA-1 (iii), and Gel-MA/Pec-CA-2 (iv), and CA (v).

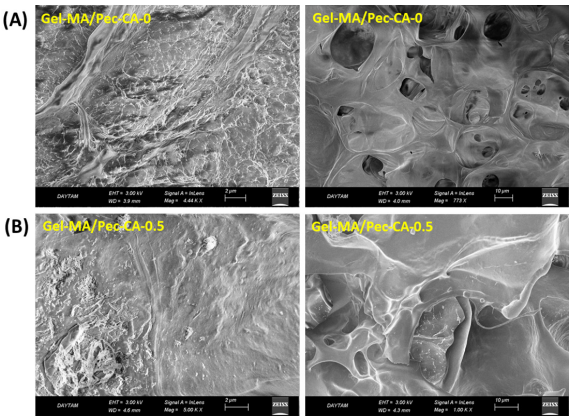


Figure 2. SEM micrographs of the surface of Gel-MA/Pec-CA-0 and Gel-MA/Pec-CA-0.5 hydrogel dressings (Scale bar: 2 μm, and 10 μm).

Water uptake ability of biomaterials is an important criterion for ideal wound dressing materials [44]. The swelling properties of the dressing contribute to a moist condition that can support wound healing [45]. The swelling degree of 3D printed wound dressings was illustrated in Figure 3-A. The water absorbing capacity of dressings was ~8 times higher than their dry weight. The swelling equilibrium of the dressings was achieved for all formulations at 24 hours (8.29±0.06, 8.22±0.09, 8.33±0.09, and 8.26±0.03 g/g for Gel-MA/Pec-CA-0, GelMA/Pec-CA-0.5, Gel-MA/Pec-CA-1, and Gel-MA/Pec-CA-2, respectively). No significant differences in the swelling degree results were found to be between 24 and 48 hours. Compared to Gel-MA/Pec-CA-0 as control, CA incorporated samples showed a similar trend in swelling behaviors. According to the literature, the moist environments in the wound area enhance the healing rate compared to dry environments during the wound healing action [46].

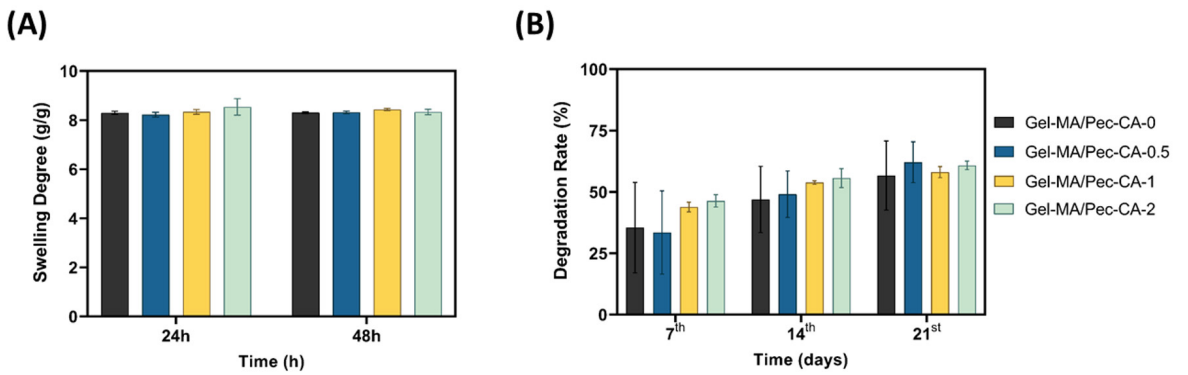


Figure 3. Swelling behaviors of the Gel-MA/Pec-CA hydrogel dressings after the immersion in PBS pH 7.4 at 37 °C (A), and degradation rate after 7th, 14th, and 21st days of incubation in PBS pH 7.4 at 37 °C (B) (n=4, ±SD).

persed homogeneously throughout the polymeric network in dressings. The SEM images also revealed that the pore structure of wound dressing did not significantly change with the incorporation of CA [43].

In vitro degradation behaviors of 3D-printed wound dressing was investigated via gravimetric method. The degradation profile of the dressings was presented in Figure 3-B. On the seventh day of the test, the degradation rate

was found to be around 50% for all formulations. On the fourteenth day, the degradation rate slightly increased by the incorporation of CA into the dressing formulation. According to data, at the end of the 3rd week, Gel-MA/Pec-CA-1 and Gel-MA/Pec-CA-2 samples had a residual amount of around 60%. In the general trend of degradation behaviors, Gel-MA/Pec-CA-2 exhibited the most rapid rate compared to other samples. This might be explained by the interaction of CA and polymer matrix [16].

Mechanical properties

Mechanical properties of materials have crucial for ideal wound healing application due to experiences such as pulling, rubbing, and impact actions caused by the environment and/or body movement [47]. To explore the effect of CA amount on the mechanical behaviours of 3D-printed formulation, their elongation at break and compression strength were defined. Figure 4-A and 4-B have shown the elongation at break and compression strength, respectively. As can be noticed from Figure 4-A, there were no significant changes in elongation at break after incorporating CA extract into 3D-printed formulations. From Figure 4-B, the compression strength redused with the increase of CA extract amount in the dressings (0.5%>%1>%2). These results indicated that CA extracts acts as a potential plasticizer thereby protecting the flexibility of the potential wound dressings. Therefore, it can be understood that the mechanical properties of the incorporating 0.5% CA extract into 3D-printed formulation prepared in this study is favorable to be prefered for wound dressing application [48, 49].

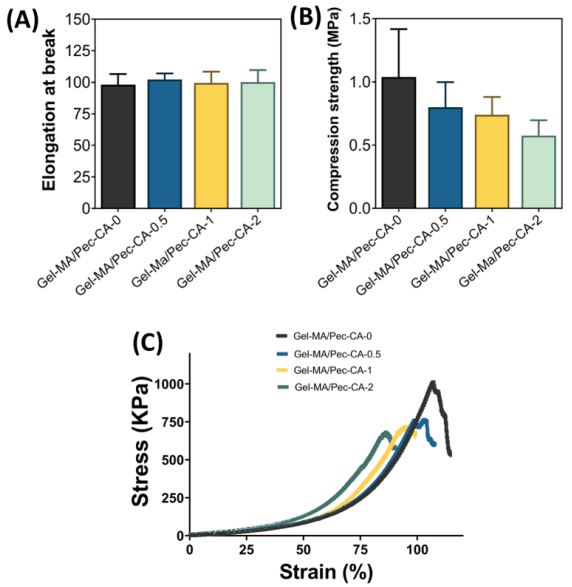


Figure 4. Mechanical properties of the Gel-MA/Pec-CA hydrogel dressings: Elongation at break (A), compression strength (B) and stress-strain curves (C) (n=4, ±SD).

In vitro bioadhesion assessment

For wound dressing materials, bioadhesion behaviour is an important characteristic for their long time usage [50]. For this purpose, the effect of the CA amount in the Gel-MA/Pec-CA wound dressings on the bioadhesive properties was investigated. The bioadhesion profile of Gel-MA/Pec-CA dressings was shown in Figure 5-A, also, Figure 5-B presented the bioadhesion force. The calculated total work of adhesion was displayed by the area under the force versus distance curve that was observed in the following order: Gel-MA/Pec-CA-0=Gel-MA/Pec-CA-0.5>Gel-MA/Pec-CA-1>Gel-MA/Pec-CA-2 [51, 52]. This result is consistent with similar bioadhesive properties of Gel-MA/Pec-CA-0 and Gel-MA/Pec-CA-0.5 dressings. However, addition of 1 and 2 wt% CA in the formulations didn't significantly affect the bioadhesive profile of CA containing wound dressings.

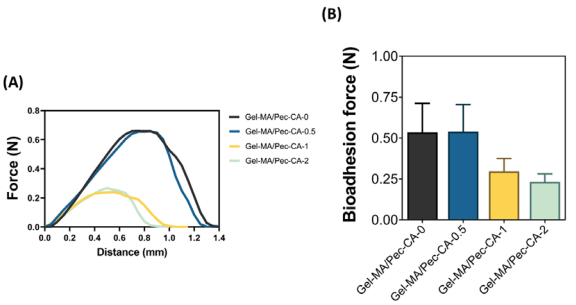


Figure 5. Bioadhesion test results of the Gel-MA/Pec-CA dressings: force vs. distance profile (A), and bioadhesion force (B) (n=4, ±SD).

Cell viability assay

The cytotoxicity of the hydrogel dressings was performed by the proliferation of NIH/3T3 cells using MTT cytotoxicity assay. As shown in Figure 6, the viability of Gel-MA/Pec-CA-0, Gel-MA/Pec-CA-0.5, and Gel-MA/Pec-CA-1 were beyond 80% compared to the negative control group after incubation for 24 h. However, cell viability results of Gel-MA/Pec-CA-2 dressing were 15% during the incubation period. Cell viability (%) below 70% is considered a cytotoxic effect according to International ISO Standard [34]. The results were found to be consistent with the literature. Gel-MA/Pec-CA-0, Gel-MA/Pec-CA-0.5, and Gel-MA/Pec-CA-1 dressings could be prefered as a safe wound dressing biomaterial because of their non-toxic effect on the NIH/3T3 cells.

CONCLUSION

In this study, 3D-printed structures had a dual croslinked network, which included UV light induced photo-crosslinking using Irgacure 2959 and psychically crosslinking with Ca^{++} ions. CA containing Gel-MA/Pec wound dressings were characterized by FTIR and SEM. Mechani-

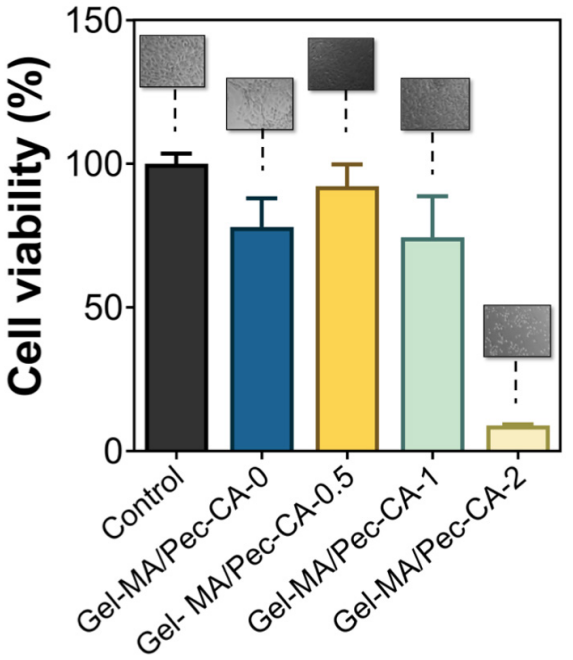


Figure 6. Cell viability of the Gel-MA/Pec-CA dressings and cell images (n=4, ±SD).

cal and bioadhesive test results presented that 0.5% CA content was the optimum amount for the usage of dressing as a wound dressing application. According to the cytotoxicity test, Gel-MA/Pec-CA-0.5 3D-printed wound dressing did not show any cytotoxic effect against NIH/3T3 cell line. In the light of this information, it was understood that Gel-MA/Pec-CA-0.5 formulation could be used for promising wound care material, which should be further investigated in future.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTION

B. Albayrak, M. Gelal and B. Izbudak carried out the experiments. A. Bal Ozturk analyzed the data. A. Bal Ozturk, B. Ozkahraman and D. Akalgan wrote the manuscript. All authors contributed to the finalization of the manuscript.

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