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PEG-STABILIZED COLLAGEN-CHITOSAN MATRICES: PHYSICOCHEMICAL AND *IN VITRO* BIOLOGICAL ASSESSMENT OF HYDROGELS

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ABSTRACT. In this study, some of the physicochemical and *in vitro* biological properties of hydrogel matrices formed under different crosslinking conditions, consisting of the structural protein collagen and chitosan polysaccharide were investigated. The chemical compositions of the developed matrices were verified, their surface morphologies were examined by SEM and AFM, and their light transmittance status was determined. Next, the *in vitro* cytocompatibility of the hydrogels was demonstrated based on one week of interaction with mesenchymal stem cells. As a result, highly transparent and cytocompatible hydrogel matrices with mechanical stability in aqueous conditions were obtained.

1. INTRODUCTION

Hydrogels are polymeric materials that can swell and maintain their three-dimensional structure in aqueous conditions. Since they have close similarities with the physicochemical properties of various tissues of the human body, they have been used in numerous biomedical applications as biomaterials [1-3]. Among the shortcomings of hydrogels are their poor mechanical properties, which are often overcome by methods such as crosslinking copolymerization, crosslinking of reactive polymer precursors, and crosslinking via polymer-polymer reactions [1,2].

Hydrogels can be of synthetic or natural origin. The lack of bioactivity in synthetic polymers has led to the use of natural-origin polymers or their combinations in the development of hydrogel biomaterials. Proteins and polysaccharides can be considered as the major classes of natural polymers. Thus, structural proteins such as collagen, gelatin, albumin, elastin, keratin,

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silk, and polysaccharides such as hyaluronic acid, chitosan, chitin, alginate are some major macromolecules of interest in the development of natural-origin hydrogels [4-7].

Studies have been carried out using collagen and collagen-chitosan to develop hydrogel matrices [6,7]. It is necessary to achieve the mechanical, biological and various other properties of the products in an application-oriented manner [7,8]. Various crosslinking methods are in use to provide the required mechanical properties. Water soluble carbodiimide, in conjunction with NHS allows, for two-step coupling of two proteins without affecting the carboxyl groups of the second protein. Homobifunctional crosslinkers, for example cross linkers with polyethylene glycol (PEG) spacers, are suitable for covalent conjugation between amine-containing molecules. This study is based upon the investigation of the physicochemical and *in vitro* biological properties of hydrogel matrices formed under different crosslinking conditions, consisting of the structural protein collagen and chitosan polysaccharide with structural similarity to glycosaminoglycans.

2. MATERIALS AND METHODS

2.1. Chemicals

Chitosan [(1-4) linked 2-amino-2-deoxy-β-D-glucopyranose] (Mr ~400,000 Da; >85% deacetylation) was supplied from Fluka (Milwaukee, WI). Collagen was isolated from rat tail tendons using the standard method. EDC [N-ethyl-N′-(3-(dimethylamino)propyl) carbodiimide], NHS (N-hydroxysuc cinimide) and all other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. NHS-PEG-NHS [polyethylene glycol bis(succinimidyl succinate)] with a molecular weight of 3400 Da was obtained from Nanocs Inc. (New York, NY). Sterile disposable tissue culture plasticware was purchased from Corning (Corning, NY). Cell culture medium, serum and supplements were obtained from Lonza (Basel, Switzerland).

2.2. Fabrication of matrices

In this study, collagen-chitosan-based hydrogel biomaterials were created using three different cross-linking methods (M1-M3). Collagen-based membranes without chitosan were fabricated as controls (C1-C3). The

compositions of the groups and the applied crosslinking methods are given in Table 1.

Collagen solution was prepared at 4°C at a concentration of 1% (w/v). Three percent (w/v) chitosan solution was prepared at 4°C with 0.2 N HCl. The collagen-chitosan-based hydrogel matrices (M1-M3) were fabricated as follows: 0.06 mL of 3% (w/v) chitosan solution and 3 mL of 1% (w/v) collagen solutions were mixed with 0.2 mL of 0.625 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer. Then, 57 µL of the cross linker solution was added and mixed well. After, the mixture was poured into disc-shaped (r= 20 mm, h= 1.5 mm) molds having a volume of 500 µL each, incubated for 16 h at room temperature, and at 37°C for 5 h, both under 85% humidity conditions. The obtained hydrogel matrices were washed in 0.1 M Na_2HPO_4 and stored at 4°C in 10 mM PBS.

For cell culture experiments, the steps were carried out under aseptic conditions and the matrices were sterilized by keeping in 70% ethanol for 6 h, and then washing with sterile PBS [9]. The contents of the cross linkers used are as follows; for M1: 57 µL of EDC/NHS (25 mg EDC and 15 mg NHS dissolved in 0.125 mL of 0.625 M MES buffer), for M2: 57 µL of NHS-PEG-NHS (Figure 1) solution (42 µL NHS-PEG-NHS dissolved in 0.125 mL of PBS), and for M3: 57 µL of EDC/NHS & NHS-PEG-NHS (37.5 mg EDC, 22.5 mg NHS, and 25 mg NHS-PEG-NHS dissolved in 0.125 mL of 0.625 M MES buffer). All conditions applied in the synthesis of the collagen-based control matrices (C1-C3) were the same as those of the experimental groups (M1-M3).

TABLE 1. Compositions of the groups

	Biopolymer		**Crosslinker**		
Groups	Col	Col-Chi	EDC/NHS	NHS-PEG-NHS	EDC/NHS & NHS-PEG-NHS
C1	+		+		
C2	+			+	
C3	+				+
M1		+	+		
M2		+		+	
M3		+			+

2.3. ATR-FTIR analysis

Structural analysis of the formed matrices was performed by using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR Spectrum 100, Perkin Elmer, Shelton, CT). The sample compartments were thoroughly dried overnight. The samples were dried and powdered finely. Spectral measurements were performed in the range of 650–4000 cm^{-1} with an accumulation of four scans at resolutions of 4.00 wavenumbers, under identical conditions.

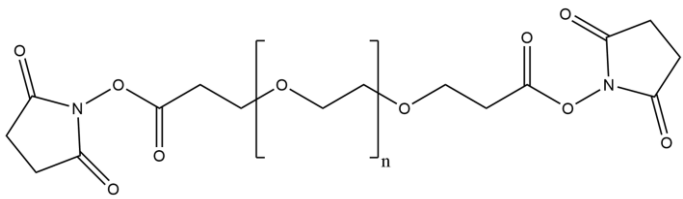

FIGURE 1. Polyethylene glycol bis(succinimidyl succinate) (NHS-PEG-NHS)

2.4. Water content analysis and stability

The difference in water contents of the matrices were determined using deionized water. The weighted equilibrium hydrated mass ($m_{hydrated}$) and dry mass (m_{dry}) values were used to calculate % water contents according to Eq.1.

$$\text{Water content (\%)} = [(m_{hydrated} - m_{dry}) / m_{hydrated}] \times 100 \quad (1)$$

To determine the mechanical stability of the matrices, samples were kept in 0.1 M phosphate buffered saline (pH 7.4) for up to 3 months and mass changes were monitored with weekly weigh-ins and Eq.2.

$$\text{Weight loss (\%)} = [(m_{initial} - m_{time\ point}) / m_{initial}] \times 100 \quad (2)$$

2.5. SEM evaluation

The surface morphology of the matrices was examined by scanning electron microscopy (SEM). Prior to examination, the samples were first lyophilized at -56ºC for 5 h, dehydrated by passing through an ethanol series (50-95%), attached to stubs and then sputter-coated with a thin layer of gold. Matrices were evaluated using the Quanta 200 FEG (Portland, OR) instrument at 10 kV.

2.6. AFM evaluation

For atomic force microscopy (AFM) analysis, biopolymer samples were formed on a mica substrate by spin coating (Primus SB15) at 3000 rpm, crosslinked by the methods described above for each matrix type, and dried in a vacuum oven at 30ºC. Analyses were carried out with a NI-AFM model (Nano Magnetics, Ankara, Turkey) device operated in dynamic mode in air using a Tap300A1 model cantilever. The surface was randomly scanned at 10μm x 10μm.

2.7. Light transmittance analysis

Optical properties of the developed matrices based on light transmission were investigated in the visible region. Matrices fabricated on glass coverslips were placed in the wells of a 12-well culture plate, and kept in PBS overnight prior to analysis [10]. The light transmittance and back-scattering measurements of the samples were carried out with a UV/visible spectrophotometer (Shimadzu, Tokyo, Japan) at room temperature. Refractive indices were measured with an Abbe refractometer (Bergman, Berlin, Germany) after the device was calibrated with alpha bromonaphthalene.

2.8. MSC culture

To determine the *in vitro* cytocompatibility of the developed matrices, human bone marrow-derived mesenchymal stem cells (MSCs; ATCC) were seeded on the matrices and cultured for up to 7 days [11]. MSCs were cultured under standard conditions; *i.e.* in α-MEM supplemented with 10% FBS, and Pen/Strep (100 U/mL penicillin, 100 μg/mL streptomycin), inside an incubator set to 37ºC, 5% CO_2, >95% humidity. Relative cell viability was assessed based on metabolic activity by the MTT (3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide) assay.

2.9. Statistical analysis

Cell viability data was presented as mean ± SD (n=3). Statistical analysis was performed via one-way Anova test followed by Tukey's post hoc test, using GraphPad Prism 7 (GraphPad, La Jolla, CA). Significance levels were set as ** $p \leq 0.01$, and ****$p < 0.0001$.

3. RESULTS AND DISCUSSION

3.1. Chemical characterization of matrices

The chemical composition of the developed matrices was evaluated by ATR-FTIR analysis, between 3800-800 cm^{-1}; findings are given in Figure 2. The formation of amide bonds between collagen and chitosan was expected as a result of different cross-linking methods applied in the study. The presence of Amide 1 and 2 bands, and also Amide 3 were monitored to confirm amide bond formations [12]. These bands show asymmetric and symmetrical stretching of NH at ~3300-3450 cm^{-1}. M3 showed a stronger peak at ~3300 cm^{-1} compared to M1 and M2. Amide 1 band stretch of C=O was observed at ~1650 cm^{-1}; H ~3050 cm^{-1} was due to the NH stretch. The band at~1530-1570 cm^{-1} was associated with Amide 2, ~1260-1300 cm^{-1} band with Amide 3, and ~2850-2960 cm^{-1} was attributed to the CH_2-CH_3 groups.

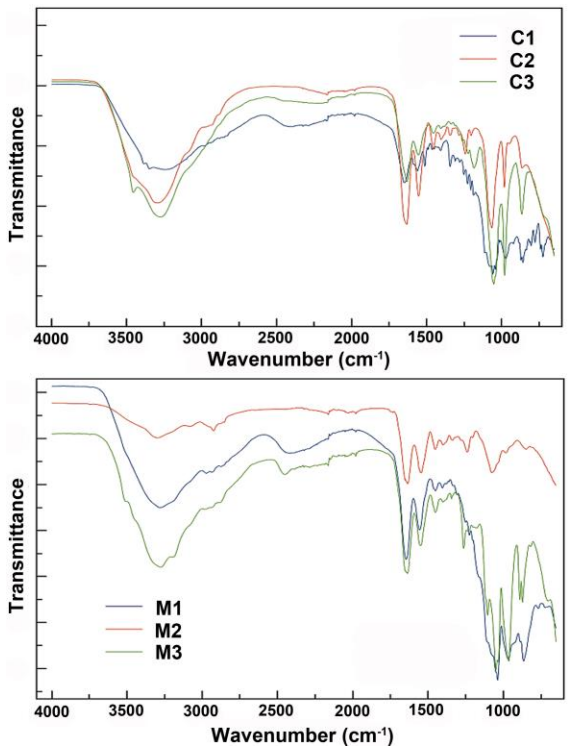

FIGURE 2. FTIR spectral analyses of (a) collagen-based control matrices (C1-C3), and (b) collagen-chitosan-based matrices (M1-M3)

3.2. Water content and stability of matrices

Water content analysis indicated that the difference between the water uptake of the matrices was insignificant (<3%). All matrices retained their structural integrity in PBS over a three-month period (data not shown). This finding indicates that collagen-chitosan matrices may be suitable for prospective long-term use in aqueous media.

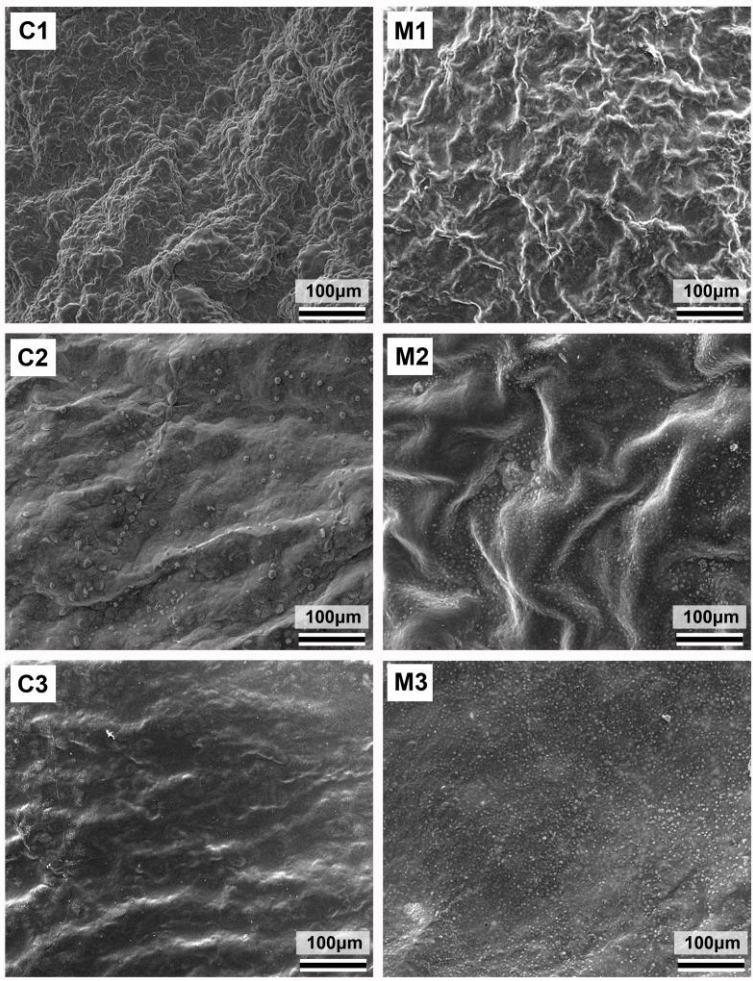

FIGURE 3. SEM evaluations of the surfaces of the matrices

3.3. Morphological characterization of matrices

Scanning electron microscopy analysis was performed to characterize the surface morphology of the developed matrices. Figure 3 contains the SEM findings of the developed matrices. Micrographs revealed that M1 and M2 type matrices (as well as C1 and C2) had rougher surface morphologies compared to M3 (also C3). M3 had the smoothest surface topography among all matrices.

Figure 4 shows high-resolution surface topographies of the matrices in the 10μm x 10μm area. The AFM images of the matrices generally revealed a homogeneous distribution of nanometer-sized (150-200 nm) clusters on the substrates.

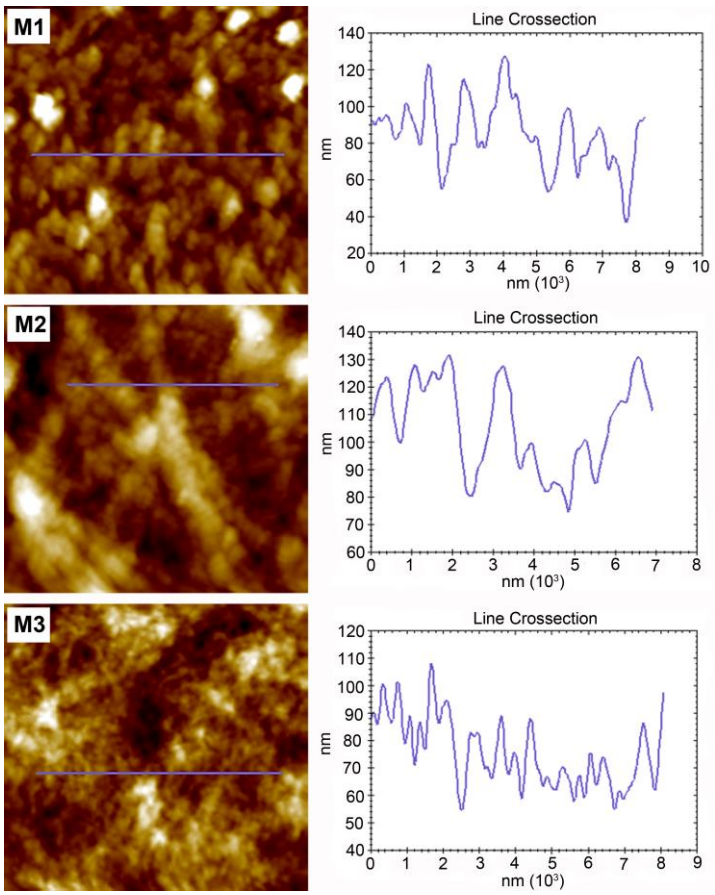

FIGURE 4. AFM cross-sectional analysis of collagen-chitosan-based surfaces

3.4. Light transmittance of the matrices

Analyses were carried out to determine the light transmittance of the developed matrices in the visible region. As can be seen in Figure 5, M2 showed the lowest light transmittance values (~50-70%) (similarly control C2, ~40-53%). Among all matrices, M3 had the highest light transmittance value of ~92-93%, compared to ~80% for M2. Transparency can be considered an important property for potential ophthalmic applications of hydrogel biomaterials, such as soft contact lenses, intraocular lenses, and ophthalmic drug delivery devices [13,14].

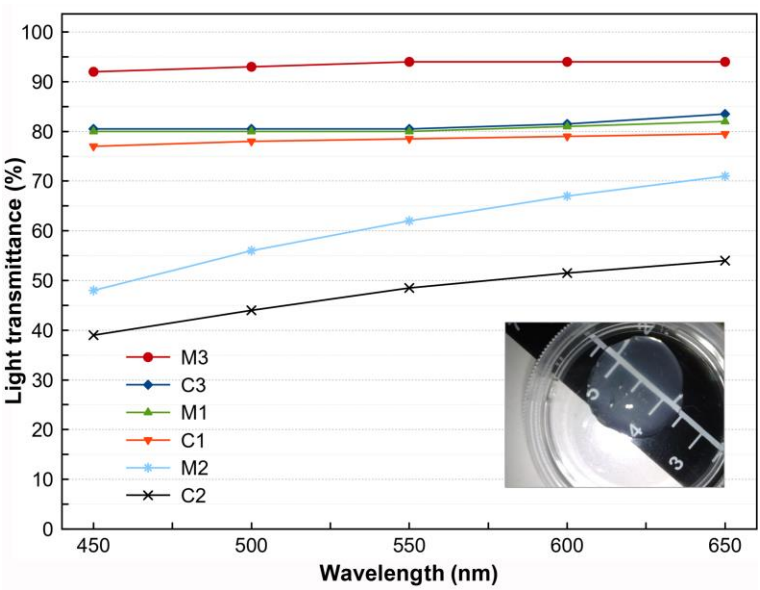

FIGURE 5. Light transmittance of the hydrogels in the visible region. Inner image: macrograph demonstrating the transparency of a collagen-chitosan hydrogel matrix

3.5. *In vitro* cytocompatibility of the matrices

To determine the *in vitro* cytocompatibility of the developed hydrogel matrices, mesenchymal stem cells were seeded on substrates and cultured for up to 7 days. The relative viability and proliferation capacity of MSCs on the matrices are presented in Figure 6. From the figure, it is clear that none of the matrices had any adverse effect on the metabolic activity of the seeded cells and the MSCs in culture proliferated over time. Relative cell viability

increased significantly between days 3 and 7 of cell culture (Figure 6). Among the groups, M3 was found to be a more suitable growth substrate for mesenchymal stem cells compared to M1 and M2.

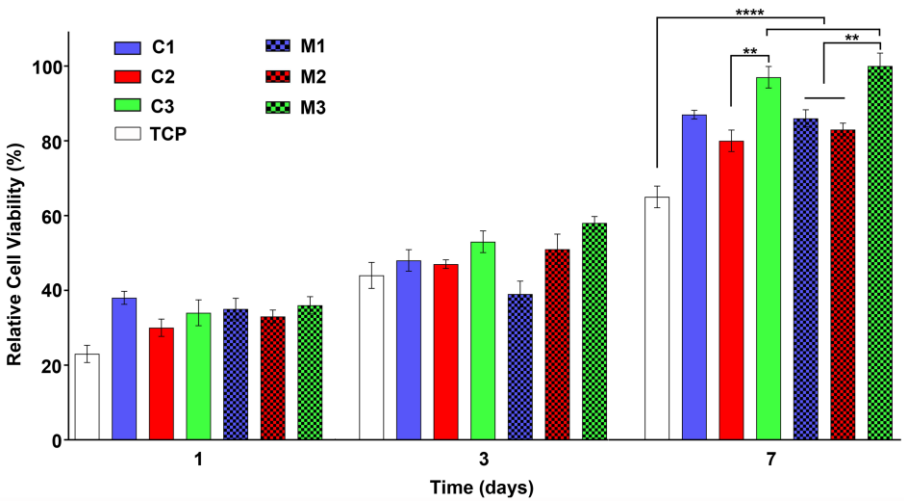

FIGURE 6. Relative viability and proliferation capacity of MSCs on the matrices assessed by MTT measurements upto 7 days in culture (**p < 0.01, and ****p < 0.0001)

4. CONCLUSIONS

In this study, collagen-chitosan-based hydrogel matrices were successfully created using three different cross-linking methods. Some physicochemical and *in vitro* biological properties of the matrices were studied in comparison with those of collagen-based hydrogels synthesized under the same conditions. All matrices retained their structural stability in a buffered aqueous environment for more than three months; this result is promising for potential future applications of matrices. Morphological analysis revealed that the formed hydrogels had relatively smooth surface features, while a smoother topography was detected for M3. *In vitro* cell culture experiments demonstrated that the substrates supported the metabolic activity of mesenchymal stem cells. The seven-day culture experiment revealed that M3 (*i.e.* PEG-stabilized collagen-chitosan) was a more suitable substrate for cells than M1 and M2. PEG-stabilized collagen-chitosan matrices also showed the highest visible light transmittance, which could be useful for

potential ophthalmic applications. However, further studies will be required to confirm this idea.

Author Contribution Statements SZ- conceptualization, investigation, data curation, formal analysis, writing. YME- supervising, resources, conceptualization, writing–review & editing. Both authors have read and approved the manuscript

Declaration of Competing Interests The authors declare no conflict of interest in relation to this particular article.

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