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Synthesis of Gum Arabic-Based Biopolymer Network and Determination of Its Toxicity Properties in In Vitro - In Vivo Model Systems

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ABSTRACT

In this study, gum arabic based network polymers were prepared using epoxy functional PEG structures. The basic physicochemical properties of these structures, their structural characterization, thermal properties and morphological properties were investigated. Toxicity properties of constructs synthesized on zebrafish (Danio rerio (Hamilton)) offspring were determined in vivo. In addition, in vitro toxicity tests were performed on L929 fibroblast cells. When the general properties of these structures were examined. Structural and thermal properties were better with increasing cross-linker rates ratios (1%, 3%, 5%). According to the toxicity test performed on zebrafish juveniles; GA-PEG-Epox (1%) constructs are non-toxic to zebrafish juveniles. The mortality rate of GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures was observed as 12.5% and 20.8%, respectively. It was observed that the structures were not toxic to zebrafish juveniles. MTT test performed on L929 fibroblast cells, high cell viability (>90%) was observed in all synthesized structures. These results are evaluated as Grade 1 according to ISO standards.

Keywords: Biopolymer network, Gum arabic, Toxicity, Zebrafish juveniles, MTT.

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Introduction

Biopolymers can be naturally occurring materials. Many materials formed during the life cycles of animals, fungi, bacteria and green plants are polymers or polymer matrix composites. Starch, cellulose are carbohydrate polymers produced by bacteria and fungi. Collagen, gelatin and silk are widely known animal biopolymers. [1]. Biopolymers are used in many areas such as pharmaceuticals, medicine, food and nutraceuticals due to their low relative density, high strength, renewable, biodegradable and inexpensive [2-4]. Biopolymers are materials of interest in many areas, such as drug release, because their thermal, electrical, and mechanical properties change when reinforced with natural substances [5]. Biopolymers such as gum arabic, chitosan, and wheat starch are commercially available, economical, and widely used examples in a variety of applications [6]. Gum Arabic (GA) is obtained from acacia wood, a type of natural polysaccharide. The structure of GA consists of galactose, arabinose, rhamnose, and glucuronic acid. Its main skeleton consists of 1,3-linked β-d-galactopyranosyl units. The presence of these units gives GA a densely branched structure [7,8]. Due to its high branching network, the hydrodynamic volume of GA is small. Thus, intermolecular interaction decreases. With its low viscosity, GA mixes easily with other ingredients without forming bubbles [9]. Determining the toxicity levels of biopolymer and polymer structures is very essential. Recently, zebrafish has rapidly gained popularity as an in vivo model for screening new materials [10,11]. Zebrafish is one of the widely used model organisms in aquatic toxicology and in vivo studies. The advantage is that these organisms are small and easy to maintain at low cost [12]. Zebrafish is widely used in determining the toxicity of water contaminants [13]. The molecular and cellular mechanisms in the nervous system development of zebrafish and its response to toxins are similar to mammalian organisms [12]. In addition to its utility advantages, we can add its high fecundity and the fact that its genome shares more than 70% of its genes with humans when compared to more complex organisms [14-16]. 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) test which is a widely used method, was used to determine in vitro cytotoxicity. The MTT assay is a colorimetric assay of living cells [17]. *Mus musculus* mouse fibroblast cells (L-929) were used in the MTT test. There are studies on GA based biopolymer structures in the

literature. Ibrahim et al. synthesized a combination of GA and polyethylene glycol dimethacrylate (PEGDMA), a synthetic polymer [18]. Amalraj et al. produced BPEO and GEO based biocomposite films containing chitosan, gum arabic and PEG [19]. Namasivayam et al. synthesized gum arabic, polyethylene glycol grafted iron oxide nanocomposite (GA-PEG-IONC) [20]. In this study, unlike the literature, gum arabic-based network polymers were prepared for the first time using epoxy functional PEG structures. It is known that there is a strong interaction between the epoxy and PEG phases in the interfacial region [21]. In this way, PEG-Epox structures were obtained. PEG-Epox structures were cross-linked with GA to obtain the GA-PEG-Epox structure. Finally, the toxicity of the prepared constructs was investigated both in vivo (zebrafish juveniles) and in vitro (L929 fibroblast cells).

Materials and Methods

Within the scope of the study, gum arabic (GA) and polyethylene glycol (PEG 1000) used in the synthesis of cross-linked polymeric structures were obtained from Sigma Aldrich. Epichlorhydrin was obtained from alfa eiser company. K_2CO_3, NaOH and other solvents used were purchased from Merc. High glucose Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin (Pen-Strep) and Phosphate Buffer (PBS) were obtained from Capricorn Scientific. Dimethyl sulfoxide (DMSO) and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich. The chemicals used in the study were purchased directly and used pure. Pre-purification was not carried out. The drying process of the synthesized gum arabic-PEG-Epox polymeric structure was carried out with Biobase brand BK-FD12S(-80℃) model lyophilizer. Chemical characterization of the synthesized structures was investigated using Perkin Elmer Spectrum Two model fourier-transform infrared spectroscopy (FTIR). FTIR spectra were taken in the 400-4000 cm^{-1} working range and the data were provided by the ATR technique. 4 cm^{-1} wavenumber measurement sensitivity was used in FTIR analysis. Characterization was also supported by nuclear magnetic resonance (NMR) spectra. NMR spectra were taken on Bruker Ultra Shield 300 MHz NMR spectroscopy. DMSO-d6 was used as the solvent. The thermal properties of the obtained structures were realized by using differential thermal analysis (DTA), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) techniques. TGA analyzes were used to determine the thermal stability of the structures and the results were determined with the Shimadzu TGA-50 model device. Analyzes were carried out at 500 ℃ in air atmosphere by taking 10 mg sample. The thermal stability and structural stability properties of the obtained networked polymeric structures were determined using the Shimadzu DTA-50 model device. DTA analyzes were performed in the range of 20-400 ℃. Shimadzu DSC-60 model device was used in the range of 20-500 ℃ to examine the softening temperatures and general structural properties of the polymers. In DSC analyses, 5 mg of sample was analyzed in aluminum cuvettes and these analyzes were carried out in a constant air atmosphere. In all thermal analyzes, the measurements were taken at a heating rate of 10℃/min. The surface and morphological properties of the obtained network polymers were determined using the Leo-Evo-40 XVP model scanning electron microscope (SEM). During SEM analysis, 10 nm Au-Pd coating was carried out using Bal-Tec SCD 050 brand spater to ensure conductivity on the samples. ESCO brand carbon dioxide incubator was used in cell culture studies.The surface, morphology and cavities of all samples were examined in detail at three different magnifications. Zebrafish juveniles and L929 cell lines were used to determine the toxicity properties of gum arabic-based networks both in vivo and in vitro, respectively.

Synthesis of Polymeric Structures

GA is a polysaccharide of plant origin and has a high molecular weight [17]. It is in the polysaccharide class due to its general structure and is used directly in many biomaterial designs. However, structurally, gum arabic type polymers are polymeric structures with very poor biopermanence. These properties limit the usage options of these polymers [22]. In the scope of the study, in order to eliminate this disadvantage, cross-linking of polymers with biocompatible PEG structures was carried out. This crosslinking process was carried out by a two-step procedure. First of all, diglycidyl ether structures were prepared using PEG 1000 and epichlorhydrin [23]. In this process, firstly, PEG 1000/epichlorhydrin at a rates of 1:2 (mole) was taken into a two-necked balloon. K_2CO_3 [~0.1% (w/w)] was added to the existing medium. THF was preferred as the solvent. The reaction was carried out at 90°C for 3 hours. After addition of calculated PEG 1000 and epichlorhydrin to a two-necked flask, the reaction was continued at 90°C for 3 hours. THF with the addition of ~0.1% (w/w) K_2CO_3 to the medium. After the reaction, it was first checked whether the PEG-Epox structure was obtained with the FTIR technique.

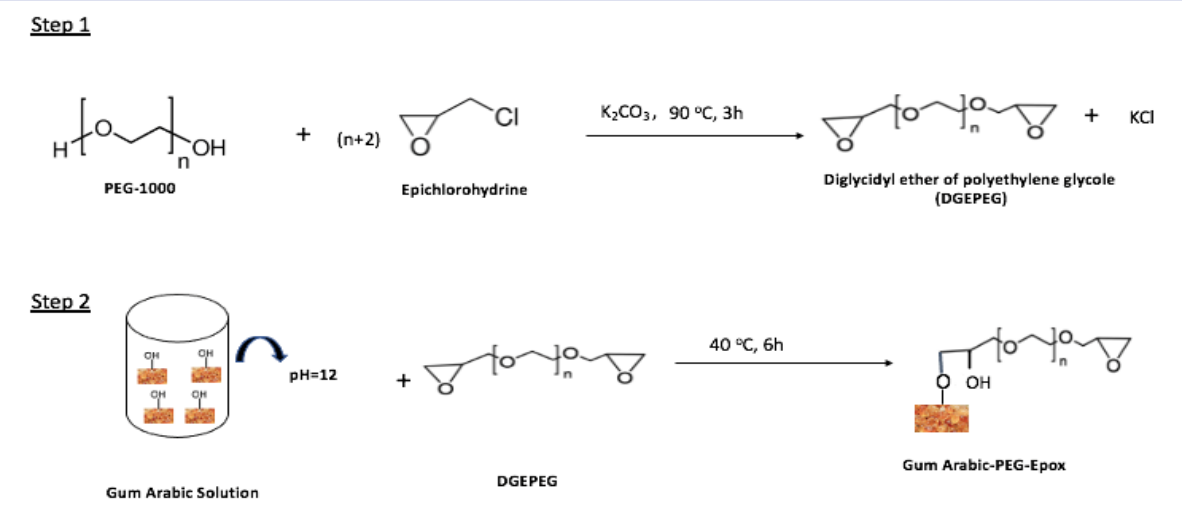

Figure 1. Synthesis of Gum Arabic-PEG-Epox (GA-PEG-Epox).

After obtaining the PEG-Epox structure, the second step was started. In the second step; The pH of the gum arabic (~ 2 mM) structure prepared in water was adjusted to pH 12 using 0.25 M NaOH solution. Gum arabic, whose pH was adjusted, was reacted with PEG-Epox at different rates (1, 3 and 5%) at 40 ℃ for 6 h under reflux. Briefly, in this second stage of the study, cross-linking of gum arabic structures was carried out using diglycidyl ether PEG 1000 structures at different rates. GA-PEG-Epox structures obtained after processing were dried using a lyophilizer. The reaction steps are given in Figure 1. The structures obtained after the reaction were confirmed by FTIR and NMR techniques.

Determination of In Vivo Toxicity Properties of Synthesized Structures on Zebrafish Juveniles

Adult AB zebrafish were grown in the Environmental Toxicology Laboratory of the Department of Biology, Faculty of Arts and Sciences, İnönü University. These fish were reared in the zebrafish aquatic system (28°C, 14:10 hours light:dark photoperiod). The system under study has automatic controls for water circulation at pH (7.30), temperature (28.5 ℃), conductivity (720 µS/cm), and light-dark photoperiod (14:10 hours). Zebrafish fry is produced in the system connected to the filtered zebrafish water system (iSpawn, Tecniplast, Italy). The grown embryos were collected within 3 hours and washed with E2 medium. Then, they were placed in petri dishes, according to Westerfield [24]. The developmental stages of embryos were identified according to Kimmel et al. [25]. Solutions containing gum arabic, gum arabic-PEG-Epox (1%), gum arabic-PEG-Epox (3%) and gum arabic-PEG-Epox (5%) in E2 medium water at 100 mg/L^{-1} was prepared. Embryos were exposed to 250 µL (pH 7.3) solutions in 96 well microplates. One embryo was added to each well of microplates. Embryos were monitored using a stereo microscope for 96 hours with 24-hour intervals. After 96 hours, body length of the surviving embryos was determined. The dimensions of the embryos were determined with Euromex Image Focus 4.0 software.

Determination of In Vitro Toxicity Properties of Synthesized Structures on L929 Fibroblast Cells

L929 cells were grown in high-glucose DMEM with 1% Pen-Strep-10% FBS in a 5% CO_2 incubator at 37 degrees. 10,000 cells from L929 were seeded in a 96-well plate. The well plate remained in the CO_2 incubator for 24 hours. 2 grams of each material was weighed in a sterile centrifuge tube and dissolved with 2000 µL of DMEM, prepared as 1 mg/ml. Then, the samples were prepared at different concentrations between 12.5-1000 µg/mL by serial dilution method using DMEM. The waiting medium in the well plate was removed and replaced with 100 µL of the medium containing different materials and different concentrations. It was then incubated for 24 hours at 37 degrees in an incubator with 5% CO_2. MTT dye at 5 mg/mL was dissolved in PBS (pH: 7.4 in Phosphate Buffer). After 24 hours, the old medium was removed from all well plates and replaced with 90 µL of DMEM in each well and 10 µL of 5mg/ml prepared MTT in each well, 100 µL of the mixture was added and incubated in a CO_2 incubator for 4 hours. After incubation, the medium with MTT in the well plates was withdrawn and escaped. 100 µL of DMSO was added to each well in the well plates. The well plates were read with the help of spectrophotometer at wavelengths of 540 nm [26] directly after treating with DMSO.

Results and Discussion

Characterization by FTIR and NMR Techniques

Within the scope of the study, GA-PEG-Epox structures were synthesized from GA and diglycidyl PEG structures, including epoxidized PEG-1000 structures at different rates. FTIR spectra of the structures obtained are given in Figure 2 by comparing them with pure gum arabic and PEG-Epox structures. Pure gum arabic structure is a polysaccharide structure and we prove the existence of monosaccharides in GA with the presence of free -OH groups [26]. In the FTIR spectrum of the pure GA structure,

stretching vibrations of free -OH groups and hydrogen bonding are seen in the range of 3000-3600 cm^{-1}[27]. The -OH stretching is due to the property of the glucosidic ring [28]. In addition, the aliphatic C-H stretching vibration is clearly evident in the range of 2850-2950 cm^{-1}. Gum arabic structures contain free carboxyl groups. At 1430 cm-^{1}, the characteristic O-H in-plane bending band is observed for carboxylic acid [29]. When we look at the FTIR spectrum, we see the symmetric and asymmetric vibrations of the carboxyl groups, respectively, in the range of 1409-1595 cm^{-1}. It is known that the peak at 1409 cm^{-1} is caused by symmetric stretching of the carboxylic groups of uronic acid residues in the gum polysaccharide [27]. In the GA structure, we see a very wide and broad peak in the range of 900-1200 cm^{-1}, which is known as the fingerprint of carbohydrates in the literature [30]. These rather broad observed peaks represent the C-C, C-O and C-O-C stretching of the polymer backbone and the C-H, C-O-H bending modes [31]. Unlike other carbohydrate structures, since the C-OH structures in gum arabic show strong hydrogen bond interactions, the peak in the range of 3200-2600 cm^{-1} is quite wide and flat. However, since the PEG-Epox groups generally tend to bind through these -OH groups, the intensity of this peak both decreases and the peak range narrows in gum arabic-PEG-Epox structures as the cross-linking rate increases. In Gum Arabic-PEG-Epox structures, C-OH stretching vibration in the range of 1780 cm^{-1}, etheric stretching vibration around 1080 cm^{-1}, C-C aliphatic C-H stretching vibration around 1380 cm^{-1}, which comes from the gum arabic structure visible. In addition, the tension vibration of the epoxy ring at 897 cm^{-1} within the epoxy unit coming from the PEG-Epox structure is clearly seen. When the epoxy ring and PEG unit are included in the structure, the aliphatic C-H stress vibration intensifies and the C-C stress vibration intensity on the structure also increases. All these findings show that the desired Gum Arabic-PEG-Epox structures are intensified with an increasing amount of crosslinking.

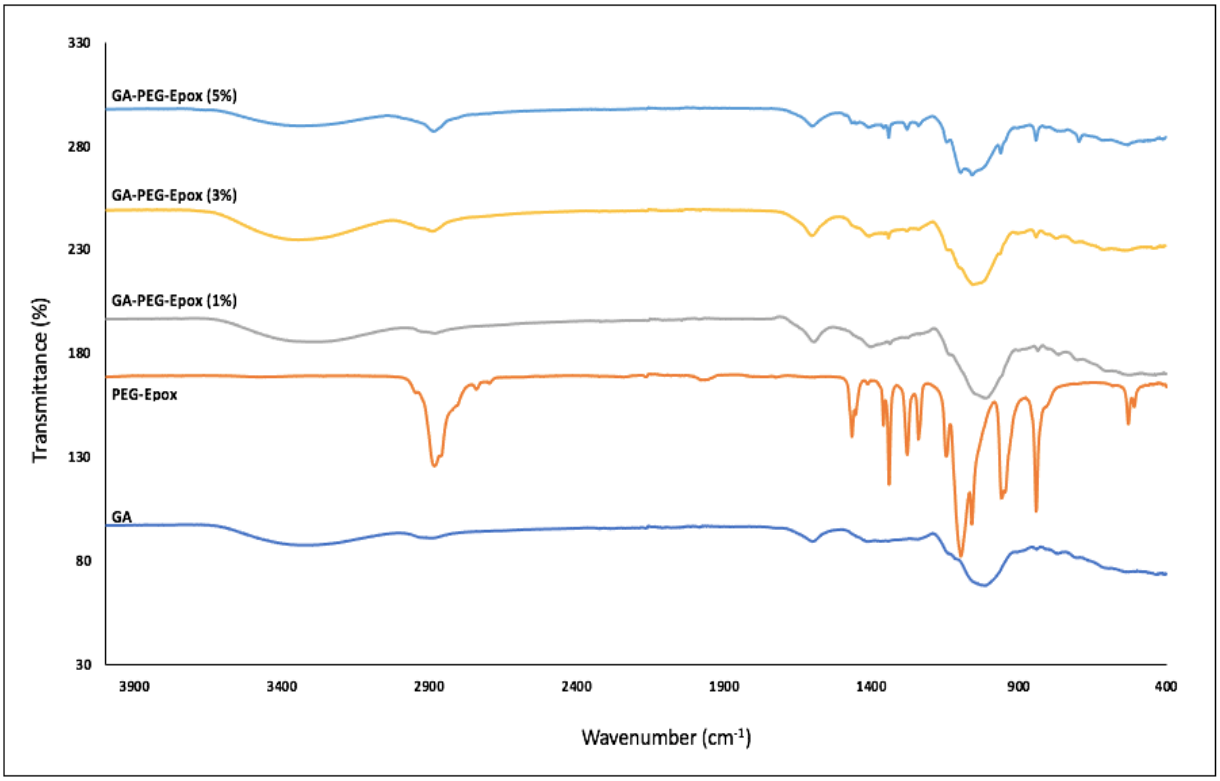

Figure 2. FTIR spectra of GA structures containing pure GA, PEG-Epox, and GA-PEG-Epox in different rates (1, 3, 5 %).

The ^{1}H NMR and ^{13}C NMR spectra of the obtained epoxy functional structures were given (Figure 3). According to the ^{1}H NMR spectra, groups such as methylene and methine in the poly(ethylene glycol) diglycidyl ether structure show similar chemical shifts. The characteristic peaks at 3.70-3.75 (m, 2H) and 3.33-3.39 (m, 2H) ppm are thought to belong to methylene hydrogen (c_1 and c_2). The peaks at 3.06–3.12 (m, 2H) ppm indicate the text hydrogen signal (b). The peaks at 2.71–2.75 (t, 2H) and 2.53–2.57 (m, 2H) ppm are due to methylene hydrogen (a_1 and a_2) (Figure 3). According to these results, epichlorhydrin is chemically bonded to both ends of the PEG chain.

In the ^{13}C NMR spectra, characteristic peaks in the poly(ethylene glycol) diglycidyl ether structure are observed. For example, after the epichlorohydrin binding of PEG, the presence of new characteristic peaks belonging to Cs in the epoxy ring is observed at 44.15 ppm (a) and 50.73 ppm (b) in the ^{13}C NMR spectrum (Figure 3).

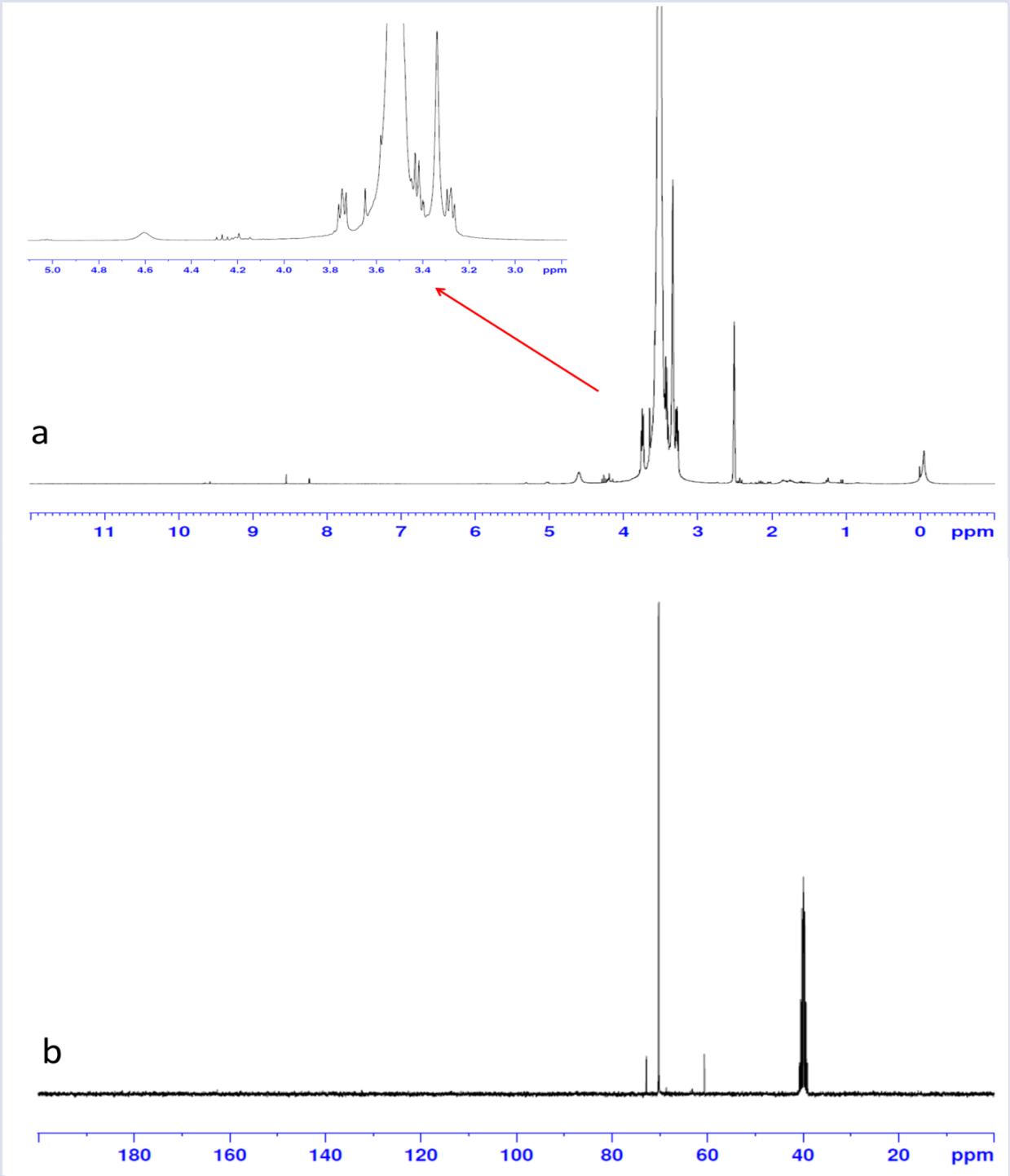

Figure 3. ^{1}H NMR (a) and ^{13}C NMR (b) results of PEG-1000-Epox

Investigation of Thermal Properties

The DTA thermograms of the obtained structures are shown in Figure 4. Gum Arabic-PEG-Epox structures were prepared with three different crosslinking rates and biopolymers with different structural stability were obtained. The thermal properties of these structures were first investigated with DTA thermograms. The thermal structures of 3 different crosslinkers are given in Figure 4 comparatively. There are 4 basic exotherms in the structure of Gum arabic-PEG-Epox 1%. 1. exotherm; between 180-200 ℃, 2.exotherm; between 220-290 ℃, 3rd exotherm; it appears prominently between 300-340 ℃ and the final exotherm between 340-380 ℃.

It is due to the PEG-Epox cross-linking units bound to the first exotherm structure, and this peak intensifies as the amount of PEG-Epox in these structures increases. The second exotherm is caused by the thermal degradation of the side group units in the gum arabic structure. The third exotherm is due to thermal degradation of the main chain gum arabic structure. The final exotherm is due to the carbonization of the decay products.

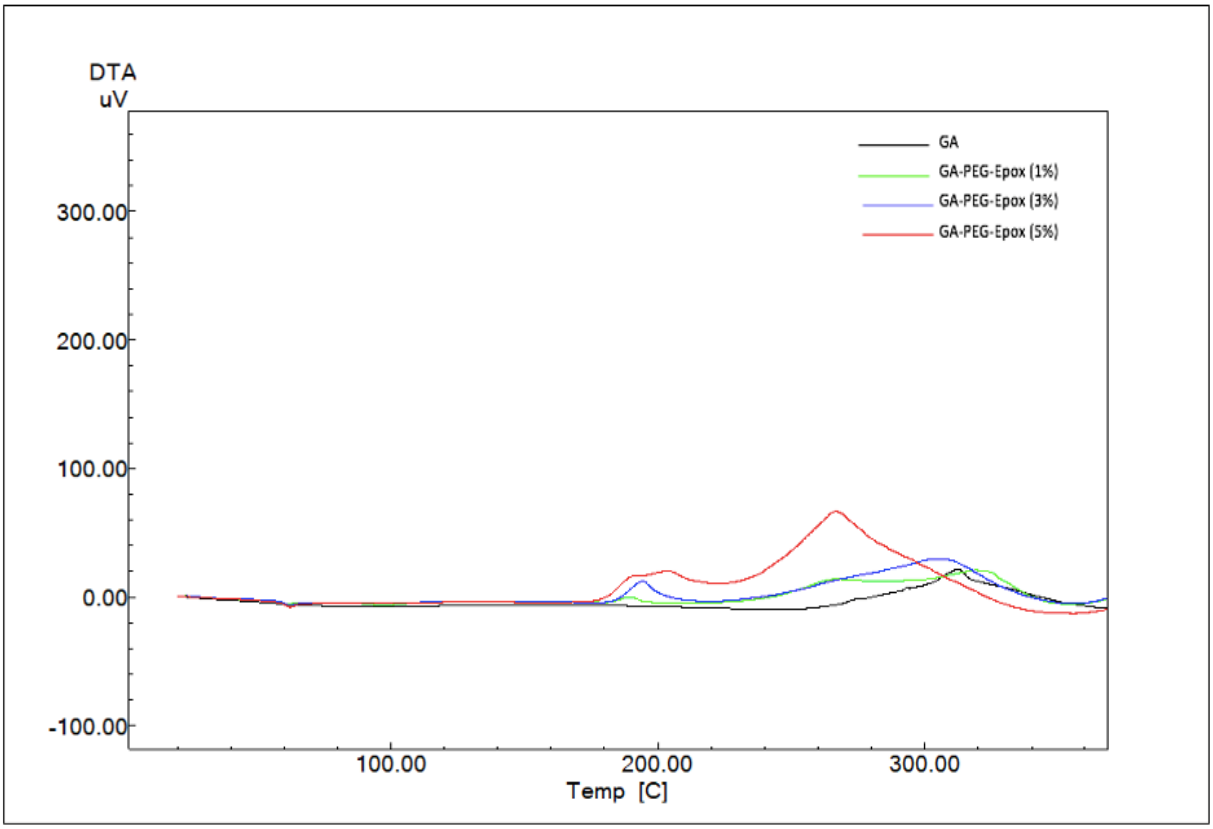

Figure 4. DTA thermograms of GA, GA-PEG-Epox (1%), GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures.

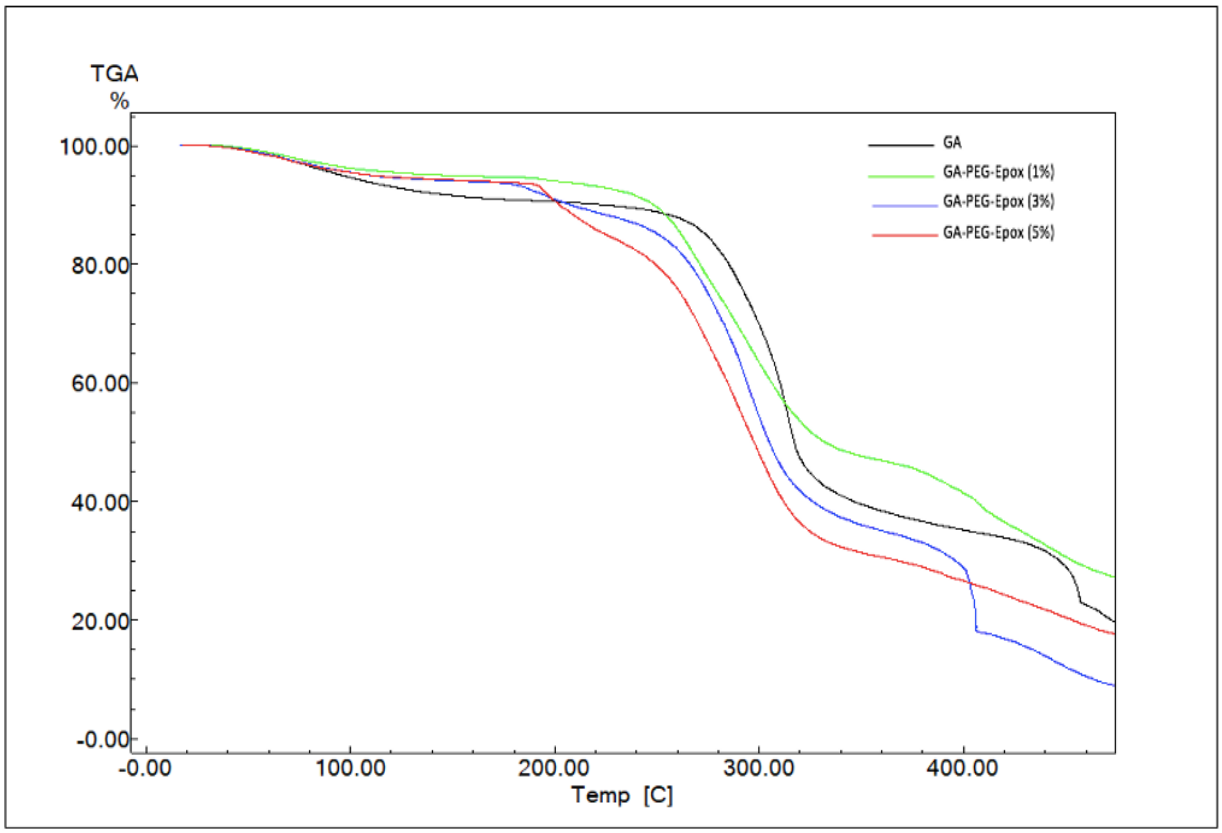

Figure 5. TGA thermograms of GA, GA-PEG-Epox (1%), GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures.

According to these DTA thermograms, the structural stability of PEG-Epox structures appears to be around 190°C. TGA analyzes were carried out between 20 and 500°C temperatures to see the related structural stability in more detail. TGA analyzes of the related structures are given in Figure 5.

In the TGA analysis of pure Gum Arabic and Gum Arabic-PEG-Epox containing different rates of PEG-Epox, basically 3 different mass losses are observed. 1. mass loss ; it is the loss of mass due to the removal of structural moisture, which appears around 20 and about 200 °C. Nep and Conway emphasized in their study that this mass loss is due to the loss of structural water adsorbed in the gums. In addition, the loss of mass is attributed to the desorption of moisture as hydrogen bonded water to the polysaccharide structure [32]. 2. the loss of mass is defined as Zohuriaan and Shokrolahi polysaccharide decomposition [33]. 2. mass loss; these are the mass losses that appear to be around 80%, resulting from the degradation of the side groups and epox units in the gum arabic structure. The final mass loss is the mass loss from the carbonization of the decomposition products, seen between 350-450°C and consistent with the DTA thermograms. When TGA thermograms are examined, we see that as the crosslinking rate in the structure increases, the thermal stability increases partially because a more rigid and more complex structure is formed. The most important reason for this increase is the cage effect. The increased thermal stability is the proof that the desired structures are obtained at the desired rate. DSC thermograms of the structures obtained are shown in Figure 6.

Endotherm and three main exotherm peaks are seen around ~80°C in these thermograms. Daoub et al. stated in their study that endothermic peaks are caused by the loss of water content in the gums, and the exothermic peaks correspond to the decomposition of the gums [34]. First exotherm ; it is the decay peak of the groups originating from PEG-Epox structures, which is also compatible with DTA thermograms and increases as the crosslinking rate increases. Second exotherm; it originates from gum arabic thermal degradation and is a fairly large peak. The last exotherm is the exotherm caused by carbonization between 360-450°C. The endotherm, around 65°C, corresponds to the softening temperature of these structures and was observed in the same temperature range in all structures.

SEM analyzes were performed to determine the surface properties and morphological structures of the GA-PEG-Epox structures obtained within the scope of the study. In these analyses, the pore structures of the obtained structures were investigated.

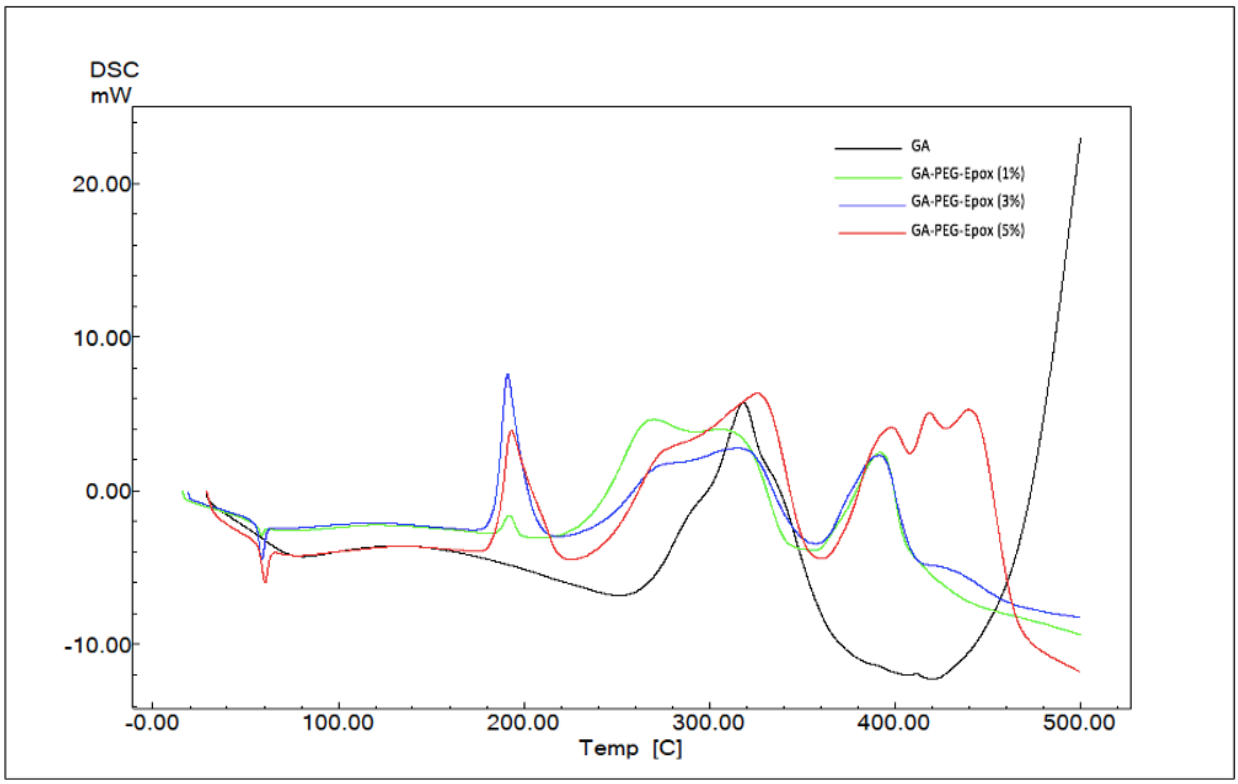

Figure 6. DSC thermograms of GA, GA-PEG-Epox (1%), GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures.

Investigation of Surface Morphological Properties

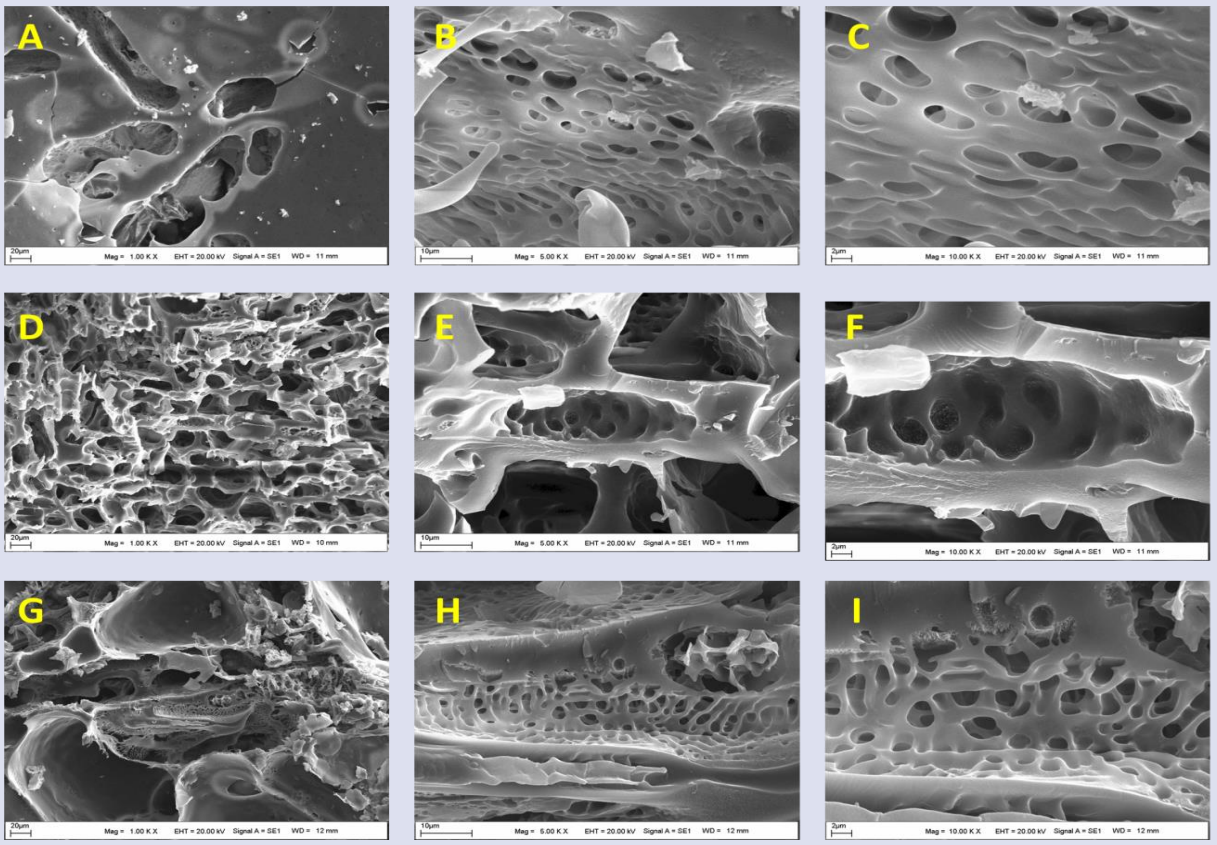

Figure 7. SEM images of the GA-PEG-Epox (1%) A (1.00 KX), B (5.00 KX), and C (10.00 KX), GA-PEG-Epox (3%) (D (1.00 KX), E (5.00 KX), and F (10.00 KX) and GA-PEG-Epox (5%) (G (1.00 KX), H (5.00 KX), and I (10.00 KX) structures.

The SEM images obtained at different magnifications are given in Figure 7. When the SEM images of GA-PEG-Epox (1%), GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures are evaluated, there are pores of different sizes and distributions in all three structures. However, it is clearly seen in Figure 7D that the GA-PEG-Epox (3%) structure has a more regular pore structure than the other structures. The pores in this structure are dense, similar in size, and distributed throughout the structure. Therefore, it shows a more ideal absorbent appearance. It is known that pore size and homogeneous distribution are very effective on absorption. Wang et al. observed that heavy metal absorption was higher in the structure with a more distinct and homogeneous pore structure [35].

Evaluation of Toxicity Properties of Synthesized Structures on Zebrafish Juveniles

The results showed that GA and GA-PEG-Epox (1%) solutions at the concentration used did not cause mortality in zebrafish. 0.1 mg/L^{-1} GA and GA-PEG-Epox (1%) are non-toxic to zebrafish juveniles. However, mortality was observed in solutions GA-PEG-Epox (3%) and GA-PEG-Epox (5%) at a rate of 12.5% and 20.8%, respectively. No significant differences were observed in the lengths of zebrafish juveniles exposed to all solutions when compared with the control groups (Table1).

Table 1. Lengths and mortality in Zebrafish juveniles exposed to the synthesized structures for 96 hours.

	Σ (Mortality)					Lengths (mm)
	n	24h	48h	72h	96h	
Control	24	0	0	0	0	3.24 ± 0.08
GA	24	0	0	0	0	3.18 ± 0.06
GA-PEG-Epox (1%)	24	0	0	0	0	3.29 ± 0.12
GA-PEG-Epox (3%)	24	1	0	2	3	3.14 ± 0.05
GA-PEG-Epox (5%)	24	0	0	2	5	3.08 ± 0.09

Evaluation of in Vitro Toxicity Properties of Synthesized Structures on L929 Mouse Fibroblast Cells

Mus musculus mouse fibroblast cells (L929) were used in the toxicity test of materials that are well soluble in water and performed by the direct method. Cell viability result is given in Figure 8. Cell images are shown in Figure 9. According to ISO-10993-5, more than 30% inhibition of cell viability is considered a cytotoxic effect [36]. The GA-PEG-Epox series (1%, 3%, and 5%) exhibited high cell viability (>90%) in the range of 12.5 to 1000 µg/mL. These results were also evaluated as Grade 1 according to ISO standards [36]. The GA-PEG-Epox series (1%, 3%, and 5%) have been observed that the materials are non-toxic and biocompatible.

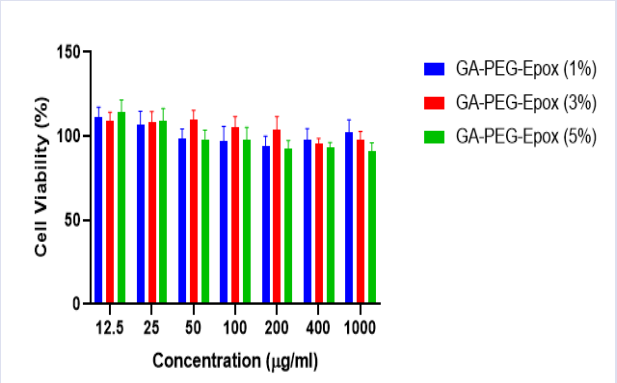

Figure 8. Cell viability results of GA-PEG-Epox (1%), GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures.

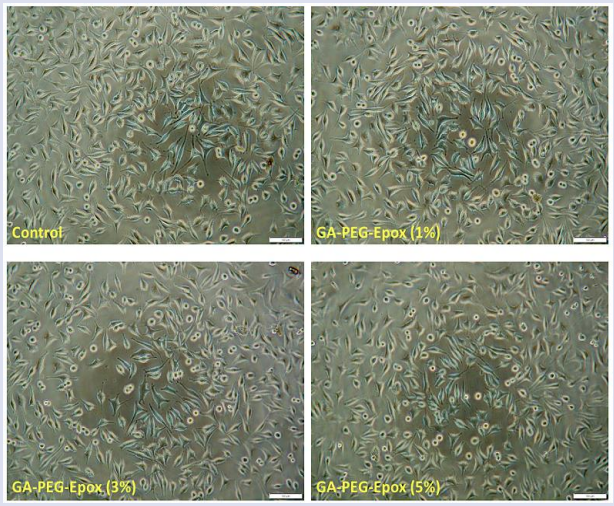

Figure 9. Cell images of GA, GA-PEG-Epox (1%), GA-PEG-Epox (3%) and GA-PEG-Epox (5%) structures.

GA, a natural polysaccharide, has a very complex molecular structure. GA contains 24%–27% arabinose, 12%–16% rhamnose, 39%–42% galactose, 15%–16% glucuronic acid, 0.22%–0.39% nitrogen, 1.5%–2.6% protein, and 12.5%–16% moisture. GA is widely used in the pharmaceutical, medical, cosmetic, and food industries. Especially in recent years, GA has been used as an effective auxiliary material in biomedical applications, drug delivery, and the realization of nanostructure scaffolds [37]. In addition, it is widely used in heavy metal removal. Abreu et al. used gum arabic to increase the adsorbent potential of chitosan-based nanoparticle structures for heavy metal removal [38]. Errich et al. performed toxic heavy metal removal using gum arabic-modified hydroxyethyl cellulose and hydroxyapatite [39]. Saeedi-Jurkuyeh et al. used PEG in the modification step to remove heavy metals from aqueous solutions [40]. It is thought that the non-toxic, biocompatible GA-PEG-Epox series we synthesized in this study can be used as an alternative design to other designs, especially for the removal of heavy metal ions from water sources.

Conclusions

In this study, network polymers were prepared by using gum arabic based epoxy functional PEG structures, which is an important natural polysaccharide. Preparation took place in two steps. In the first step, diglycidyl ether structures were prepared using PEG 1000 and epichlorhydrin. The process was followed by the FTIR technique. After obtaining PEG-Epox, gum arabic was reacted with PEG-Epox under a reflux system. After reaction, GA-PEG-Epox series (1%, 3% and 5%) were obtained. Structural characterization was elucidated by FTIR and NMR techniques. Thermal properties were determined by TGA, DTA and DSC techniques. Surface morphology was elucidated by SEM technique. In vivo and in vitro toxicity tests were performed on zebrafish juveniles and L929 fibroblast cells, respectively. All findings proved that GA-PEG-Epox(1%, 3%, 5%) serial network polymeric structure was synthesized and non-toxic. In future studies, whether non-toxic GA-PEG-Epox (1%, 3%, 5%) network polymeric structures can be an alternative adsorbent for removing heavy metals, an essential problem in water resources, will be studied in detail.

Conflicts of interest

There are no conflicts of interest in this work.

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