

# Synthesis and Evaluation of Gelatin Methacrylate/Poly (2-Ethyl-2-Oxazoline) Porous Hydrogel Loaded with Kartogenin Drug as a Biocompatible Scaffold for Cartilage Tissue Regeneration

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**Abstract:-** Porous hydrogels can serve as excellent scaffolds for cartilage tissue regeneration to bridge the defect gap because if the cartilage is damaged, it has a limited ability to self-regenerate. Tissue-engineered scaffolds are often an ideal medium for repairing, replacing, or regrowing lost or damaged tissue. These scaffolds act as extracellular matrix (ECM) in nature and are required to promote cell development and differentiation. The hydrogel approach is a quick and effective way to create scaffolds from biocompatible polymers among scaffold fabrication techniques. In this research, via synthesis of Gelatin Methacrylate (GelMA) and Poly(2-Ethyl-2-Oxazoline) (PEtOx) along with loaded Kartogenin drug (KGN), a porous hydrogel with an advanced structure was created to regenerate the extracellular matrix of cartilage. Evaluations were made on morphology, swelling ratio, degree of hydrophilicity, weight loss percentage and degradation, elastic modulus, blood compatibility, drug release, flow cytometry and MTT. The data revealed that the created hydrogel was quite porous, with a porosity of 92.2% and linked pores. With a weight loss of approximately 43% within 14 days, it was found that the produced hydrogel is biodegradable at the same time as cell proliferation. After the stress/strain test, we found that the elastic modulus of the GelMA/PEtOx hydrogel with the highest percentage composition and maintaining the structure and morphology is close to the elasticity modulus of the cartilage and also the prepared hydrogel showed a good blood compatibility and sustainable drug release. According to flow cytometry and MTT tests, up to 75% of cells can live in this hydrogel and create cell adhesion.

**Keywords:-** Cartilage, biocompatible hydrogel, Gelatin Methacrylate, Poly(2-Ethyl-2-Oxazoline), Kartogenin.

## I. INTRODUCTION

Only a small number of chondrocyte cells can be found in articular cartilage tissue because cartilage does not have blood vessels [1]. Water, collagens, proteoglycans, and noncollagenous glycoproteins make up the ECM of cartilage, which gives the tissue its compressive strength and permits frictionless motion during loading. Water molecules account for 80% of the weight of articular cartilage, sustaining its flexibility and lubricity and acting to transfer nutrients [1, 2]. These processes affect the rheological viscoelastic characteristics of cartilage when it is subjected to a constant load [2]. Since articular cartilage has a poor capacity for regeneration and many clinical treatments are not available, tissue engineering has emerged as a possible alternative [3].

Hydrogel-based scaffolds have received a lot of interest recently in cartilage tissue engineering in cartilage regeneration and so far several natural and synthetic biocompatible polymers have been used for synthesis of hydrogels [4]. One of the famous derivatives of gelatin is Gelatin Methacrylate (GelMA). Bioactive motifs in gelatin, including arginine-glycine-aspartic acid (RGD), help different types of cell adhere to each other and grow. GelMA is a natural polymer and has less than 5% methacrylic anhydride [5], and is created by replacing the free amine groups of gelatin with Methacrylic Anhydride (MA) and at the same time, it leaves intact the RGD sequences that are inherent in natural polymers and increase cell adhesion and final degradation of the scaffold. Gelatin acquires the ability to photocrosslink when exposed to UV radiation because of the insertion of methacrylate substituent groups and photoinitiator [6]. GelMA hydrogel has gained popularity due to its ability to form a stable gel at body temperature (37°C) [7].

A well-known synthetic polymer often used in biomedicine is polyethylene glycol (PEG). Recent research has shown that PEG with gelatin promotes chondrocyte-mediated remodeling and increases the production of articular cartilage matrix (Sridhar et al. 2015). Nevertheless, several limitations in the synthesis of PEG-based hydrogels have been reported. PEG dissolves in solid and hydrogel-like forms as well as in dilute solutions [8]. Anti-PEG antibodies have often been seen in some patients who have received injectable GelMA/PEG hydrogel, which eventually caused anaphylactic shock due to deposition in blood vessels. Therefore, we decided to investigate and analyze Poly(2-Oxazoline) (POx) to find out if POx can replace PEG in GelMA/PEG hydrogel by maintaining the same properties but with very low toxicity. In this study, we used the most common commercial type of POx, Poly(2-Ethyl-2-Oxazoline) (PEtOx) for in-vitro analysis. PEtOx are pseudo peptides that can be made via live Cationic Ring-Opening Polymerization of 2-Oxazolines. Microwave radiation can help PEtOx synthesis. Since the tertiary amide groups in PEtOx are complex to be recognized and hydrolyzed by enzymes, the stability of the molecule in natural settings is increased [9, 10]. On the other hand, the connections of reactive and functional groups to the end of the chain and polymer side groups in PEtOx can be an ideal candidate for making hydrogel [11].

By incorporating the drug into the polymer chain, scaffold-drug conjugates have been investigated as a biomedical method to increase the treatment and their simultaneous transport to the target tissue by taking advantage of the enhanced permeability and retention effect [11]. Kartogenin (KGN), a small molecular drug, can promote chondrogenic development in human mesenchymal stem cells, releasing core-binding factor, and binding to transcription factor RUNX1 during mesenchymal stem cells differentiation. As a result, KGN has a significant potential for application in cartilage regeneration [12]. KGN should not be injected directly because it is quickly absorbed through the bloodstream in non-targeted tissues. However, its other applications may be restricted by the hydrophobic characteristic of KGN and underlying unfavorable reactivity to untargeted tissues. Therefore, KGN should be loaded into a drug delivery system in order to both overcome the hydrophobicity of KGN and deliver the drug directly to the target tissue, preventing drug diffusion and deposition at non-targeted sites [13].

In the present study, we have conducted an in-vitro investigation to examine the properties of GelMA/PEtOx hydrogel loaded with KGN drug. To assess the morphology, biocompatibility, and biodegradability of the hydrogel, we

have employed FT-IR and SEM images, analysis of swelling, contact angle, and water loss. In order to approximate the elasticity modulus of cartilage, we have prepared GelMA/PEtOx hydrogel samples by combining different percentages for mechanical testing. The blood compatibility test has been employed to determine blood coagulation and hemolysis in the presence of GelMA/PEtOx hydrogel. The effective release rate of KGN drug from the GelMA/PEtOx hydrogel has been meticulously analyzed and investigated for a period of fourteen days. Finally, we have conducted cell culture and cell viability studies to determine the impact of the GelMA/PEtOx hydrogel on cell adhesion and proliferation.

## II. MATERIALS AND METHODS

### A. Materials

Poly(2-Ethyl-2-Oxazoline) (Mw= 200000 g/mol, CAS-Number 25805-17-8 (C<sub>5</sub>H<sub>9</sub>NO)) and Kartogenin drug (Mw= 317.3g/mol, CAS-Number 4727-31-5 (C<sub>20</sub>H<sub>15</sub>NO<sub>3</sub>)) were purchased from Sigma-Aldrich, USA. Gelatin Methacrylate (Mw=45000 g/mol, CAS-Number 228018-77-9 ((C<sub>40</sub>H<sub>59</sub>N<sub>11</sub>O<sub>13</sub>)<sub>n</sub>), Buffer Phosphate Saline tablet (PBS) (Mw= 94.971 g/mol (C<sub>12</sub>H<sub>3</sub>K<sub>2</sub>Na<sub>3</sub>O<sub>8</sub>P<sub>2</sub>)) and Irgacure 2959-Initiator (Mw= 224.25 g/mol, CAS Number 106797-53-9) were prepared from ZFZ Co, Iran.

### B. Preparation of Hydrogels Network

First, we created a transparent liquid by dissolving 0.15 g of GelMA powder in 1 ml of PBS. Then, for 20 minutes, the solution was thoroughly mixed on an electric stirrer at a temperature of 100°C and a speed of 250 revolutions per minute (we covered the beaker with paraffin and foil to prevent water from evaporating and to prevent light from hitting the sample). After stirring for 20 minutes, we lowered the temperature to around 20-30°C, added 10% solid weight of GelMA, Irgacure 2959 initiator, and then raised the temperature to 100°C. After 20 minutes, we reduced the temperature once again by 20°C, then we allowed the polymer solution to be completely mixed on a stirrer to obtain a gel-like state.

We weighed 0.05 g of PEtOx and dissolved it in 0.5 mL of PBS solution. 2, 6, and 10 µg/µL concentrations of KGN were sonicated in the PEtOx/PBS solution, and in the last 30 minutes of the hydrogel synthesis, to GelMA solution was added (The whole process of hydrogel synthesis is 90 minutes). Finally, the polymer solution was placed inside a microtube and exposed to UV light at a wavelength of 360 nm and a voltage of 30 V for 2-3 minutes to cross-link (Figure 1).

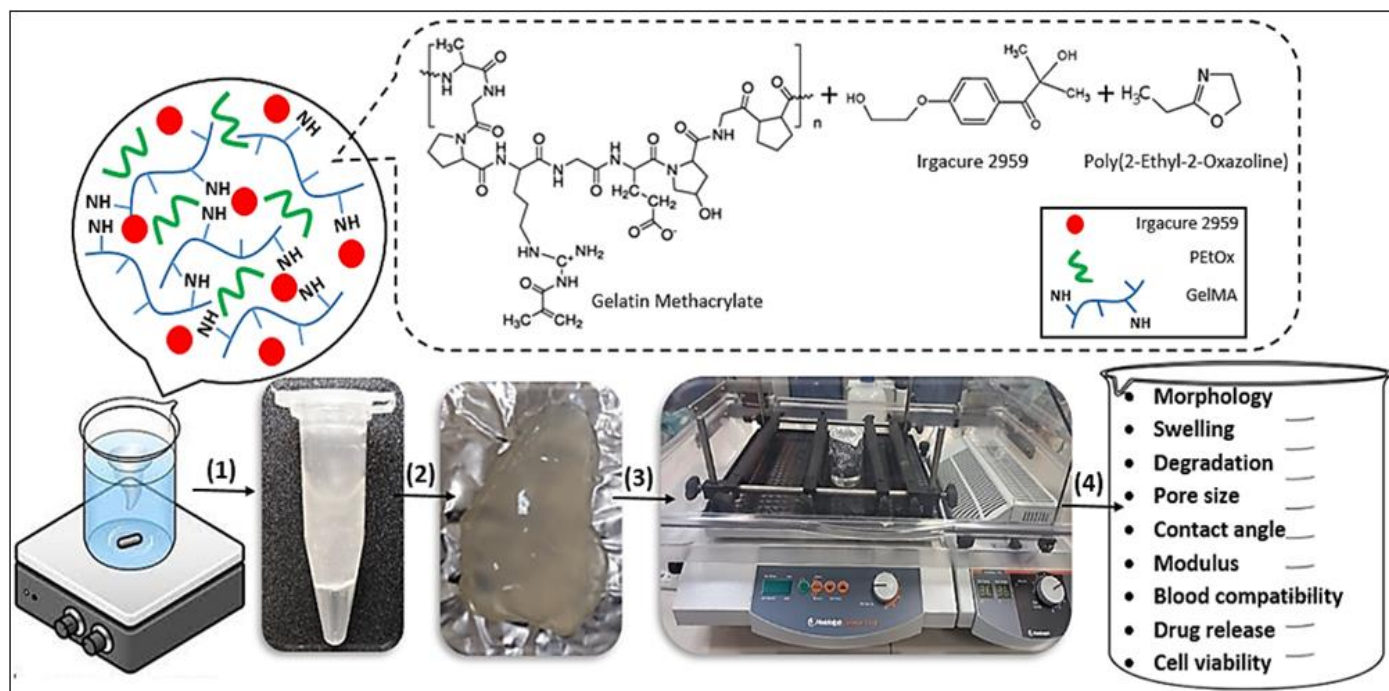


Fig 1 Scheme of the GelMA/PETox hydrogel synthesis, (1) After 90 minutes on the magnetic stirrer, hydrogel solution is poured into the microtube for place under UV light (360 nm), (2) The GelMA/PETox hydrogel picture after crosslinking under UV light for 180s, (3) Placing the hydrogel in the incubator to prevent chemical bonds from loosening and weakening before performing the analyses, (4) Perform in-vitro analyses

### C. Characterization and Analysis

#### ➤ SEM Images

To analyze the substructures of the GelMA/PETox hydrogel, an initial step was taken to freeze the hydrogel with liquid nitrogen. Subsequently, the hydrogel was dried in a freeze-dryer and meticulously coated with gold for a duration of 250 seconds. Following these steps, the hydrogel was subjected to analysis under a Scanning Electron Microscope (SEM) (SC7620-Qorum-Technologies-England) at an accelerating voltage of 20 kV. The average size of the GelMA/PETox hydrogel pores was calculated by selecting 30 random pores using Image J (NIH).[14].

#### ➤ Infrared Analysis

A recording of the Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy measurement of GelMA/PETox hydrogel was undertaken by means of a Perkin-Elmer Spectrum BX FT-IR spectrometer, spanning the range of 500-4000  $\text{cm}^{-1}$  and with an OPD resolution of 4  $\text{cm}^{-1}$ .

#### ➤ Evaluation of Swelling Ratio

In order to reach a constant gel weight, the GelMA/PETox hydrogel samples were lyophilized for 24 hours at  $-54^{\circ}\text{C}$ . This amount of dry gel was submerged in PBS solution, with a pH of 7.4, at a temperature of  $25^{\circ}\text{C}$  and put inside the incubator. Every hour, the swelling behavior was examined by removing the hydrogel from the solution and weighing it by Eq. 2-1 [15]:

$$\text{Swelling Ratio (\%)} = \frac{M_1 - M_0}{M_0} \times 100 \quad (2-1)$$

- $M_0$  and  $M_1$  is the weight of the hydrogel before and after water absorption, respectively.

#### ➤ Degradation of the Hydrogel

Whether the gels are derived from natural resources or synthesized, biodegradability is a crucial factor for many injectable biomaterials. The rate at which a scaffold degrades should ideally match the rate at which new tissue develops, and this time depends on the sort of tissue being formed [16]. The formation of new tissue may be hampered by the persistent presence of polymers at the injection site. However, for the regeneration of some tissues, such as articular cartilage or the cornea, total degradation may not be required. Gels that deteriorate to a certain extent and then merge with the local tissue to provide permanent or semi-permanent support may be appropriate for these applications [17]. The weight loss study is employed to assess the rate of degradation of hydrogel. According to this, the dry weight of the dried hydrogel was calculated, and it was then stored at  $37^{\circ}\text{C}$  inside an incubator in a Falcon tube containing PBS. The amount of hydrogel degradation was then measured by removing the hydrogel from the solution after each drying, between the seventh and fourteenth day, by Eq. 2-2 [18]:

$$\text{Degradation ratio (\%)} = \frac{W_0 - W_1}{W_0} \times 100 \quad (2-2)$$

$W_0$  and  $W_1$  is the initial weight of the dried hydrogel and the weight of the secondary dried hydrogel after taking out from the PBS solution, respectively.

### ➤ Investigating the Porosity of the Hydrogel

In the hydrogel pore structure, cells may cause pore blockage and prevent cellular penetration within the scaffold if the pore diameter is too small. Therefore, it is necessary to weigh the balance between increasing the pore size and decreasing the interior surface area. Otherwise, the mechanical strength decreases as the pore diameter increases. The amount of vacant space in the scaffolds must be precisely controlled to hold a lot of cells while preserving the structural integrity of the tissue [19]. In this analysis, we tested the hydrogel matrix in 96% ethanol to investigate its porosity. The sample was immersed in the ethanol solution and then placed in the incubator, and the porosity of the hydrogel was evaluated every 10 minutes with Eq. 2-3 [20].

$$\text{Pore size (\%)} = \frac{V_1 - V_3}{V_2 - V_3} \times 100 \quad (2-3)$$

$V_1$ ,  $V_2$  and  $V_3$  is the initial volume of ethanol, the volume of ethanol after adding the hydrogel, and the volume of ethanol after taking out the hydrogel (after every 10 minutes), respectively.

### ➤ Investigating the Contact Angle of Water with Hydrogel

Hydrogels are extremely hydrophilic due to their capacity to store a lot of water. Due to this feature, cells can grow and multiply more effectively in an environment similar to ECM. Gels are highly degradable due to their high water content. The hydrophilicity of the hydrogel is another factor that affects the ease with which the encapsulated cells or growth factors are released [17]. According to Eq. 2-4, contact angle of the hydrogel with water was measured to determine its hydrophilicity or hydrophobicity [21].

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos\theta \quad (2-4)$$

The symbol  $\gamma_{sv}$  is representative of the surface free energy of the hydrogel, while  $\gamma_{sl}$  represents the surface free energy of the hydrogel/water droplet. Furthermore,  $\gamma_{lv}$  represents the surface free energy of the water droplet, and  $\theta$  pertains to the angle at which the water droplet makes contact with the hydrogel.

### ➤ Mechanical Test

The success of the implant depends on the biomaterial used having the suitable mechanical properties for use in a tissue engineering application [22]. Natural hydrogels have been found to exhibit low mechanical characteristics and sometimes stimulate the immune system. Additionally, they are frequently regarded as weak and structurally intricate, which often limit the opportunities for hydrogel structural change [23]. The reverse is true for synthetic polymers. High mechanical qualities, including controllability in shape, mechanical strength, compressive strength, and porous structure, are features of synthetic polymer. To synthesize hydrogels for various applications in biomedical areas, natural and synthetic polymers can therefore be made utilizing polymer blending that allows the adjustment of the physical and chemical properties of both types of polymers [24]. Naturally synthesized hydrogels at various concentrations have been shown to have a considerable impact on cell adhesion, cell morphology, and cellular

differentiation. The changes in the performance of a scaffold are typically linked to the mechanical properties of the matrix because the stiffness of the matrix increases with increasing polymer concentration. The polymer concentration also has a significant impact on changes in the density of cell-matrix binding sites, as well as changes in pore size, pore shape, porosity, and permeability. Accordingly, depending on the target tissue type and its application, the effective polymer concentration for regeneration performance should be researched [7]. We synthesized four samples with varying amounts of hydrogel to test the amount of stress/strain and elastic modulus of hydrogel (Eq. 2-5). The samples contained 10% GelMA and 35% PEtOx, 10% GelMA and 45% PEtOx, 20% GelMA and 35% PEtOx, and finally, 20% GelMA and 45% PEtOx, respectively. The mechanical properties of GelMA/PEtOx hydrogels were examined by an Instron 5969 universal testing machine. The hydrogels underwent treatment via utilization of a load cell, which was preloaded to 0.06 N at ambient temperature. Subsequently, the hydrogels were subjected to a continuous pressure of 2 mm/min until fracture occurred. The elastic modulus was determined by analyzing the stress-strain curve [14].

$$\text{Elastic modulus} = \frac{\text{Stress}}{\text{Strain}} \quad (2-5)$$

### ➤ Blood Compatibility

The assessment of hydrogel compatibility with blood is a crucial analysis, as the feasibility of injecting hydrogel into the body is contingent upon this compatibility, which is determined through the utilization of equation 2-6. To conduct this examination, 2 ml of freshly anticoagulated blood from human volunteers was combined with 2.5 ml of normal saline, with 0.2 ml of diluted blood being employed for the test samples. The mixture was held at a temperature of 37°C for 60 minutes, then subjected to centrifugation four times at 1500 rpm for 10 minutes. The resulting supernatant was collected, and the purified blood was brought into contact with the hydrogel. Subsequently, light absorption was measured through a microplate at a wavelength of 545 nm. The entire experiment was performed thrice, and the average value was recorded.

The positive control group comprises of 0.2 ml of blood, which has been suitably diluted in 10 ml of deionized water. On the other hand, the negative group consists of 0.2 ml of blood, which has been appropriately diluted in 10 ml of normal saline.

$$\text{Hemolysis rate (\%)} = \frac{A_1 - A_3}{A_2 - A_3} \times 100 \quad (2-6)$$

$A_1$ ,  $A_2$ , and  $A_3$  represent the levels of absorbance exhibited by the hydrogel sample group, the positive control group, and the negative control group, respectively [25].

### ➤ Drug Release Analysis

In order to study the amount of KGN released from the gel, first the gel containing KGN was placed in a PBS solution with pH = 4.7 and a temperature of 37 °C in an incubator-shaker. At suitable time intervals, approximately 1

ml of the aforementioned solution's supernatant was withdrawn for spectrophotometry at a wavelength of 285 nm and subsequently replaced with 1 ml of fresh PBS solution. The quantity of drug present in the supernatant was ascertained through employment of the measurement kit and by utilizing equation 2-7 [26]. The control group included hydrogel without Kartogenin. To determine the rate of KGN release from the hydrogel, the methodology was executed in accordance with the Beer-Lambert law.

$$\text{Loading efficiency (\%)} = \frac{\text{Total amount of drug} - \text{Free drug in the supernatant}}{\text{Total amount of drug}} \times 100 \quad (2-7)$$

#### ➤ Flow Cytometry Analysis

A widely used analytical technique known as flow cytometry uses equipment to scan individual cells as they pass through excitation sources in a liquid solution. The technology is exceptional because it can quickly and quantitatively analyze multiple parameters for a single living or dead cell. As a result, it can be used to examine cell cycle progression, apoptosis rates, and patterns of cell death [27]. Using a hemocytometer, NIH/3T3 cells, which are several cell lines of mouse embryonic fibroblasts, were placed in the GelMA/PEtOx hydrogel cell culture medium. Trypan blue was used as an antiprotozoal drug and substantive dye for cell viability. Ten thousand cells per well of 96-well plates were planted. To attain the final polymer concentration (0.008-1 mg/mL by dilution, 110  $\mu$ L per well), the culture medium was replenished, and polymer solutions (50% v/v) were added. The cells were then cultured for 48 hours at 37°C in a humid incubator with a 5% CO<sub>2</sub> environment. A 5% v/v solution of trypan blue in DMEM was added after removing the media and incubating the cells. Another four hours were spent incubating the cells at 37°C. Fluorescence was used to measure cell viability (excitation: 540 nm, emission: 590 nm). Cells cultured with PBS without polymer were used as control sample [28].

#### ➤ MTT Assay

The scaffold should ideally be bioactive to support and direct cell proliferation, differentiation, and tissue growth without inducing an inflammatory response or activating the immune system. Since the RGD peptide sequence that exist in natural polymers, has ligands that act as receptors, it helps

cells to stick to surfaces and increase cell adhesion, and on the other hand, because they are proteins and have an enzyme structure, they can significantly contribute to the destruction of scaffold [29, 30]. The MTT test has been used extensively to evaluate cell adhesion and cytotoxicity [27]. We investigated the cytocompatibility of KGN loaded in GelMA/PEtOx on fibroblast cells from cartilaginous tissue. In a flat 96-well plate, cartilage fibroblast cells were grown with or without the KGN drug in GelMA/PEtOx. Cells were stained for live/dead on days 1, 3, and 7 following seeding [31]. The stained cell on the well was examined under a fluorescent microscope after 30 minutes of incubation. Live cells were exhibited in a verdant hue, whilst expired cells were illustrated in a crimson shade [32]. Confocal Laser Scanning Microscopy (CLMS) was used to examine cell uptake.

### III. RESULTS AND DISCUSSION

#### ➤ SEM Images

GelMA/PEtOx hydrogel was created by combining GelMA and PEtOx solution. UV light was then used to cross-link the mixture. After 2 minutes of cross-linking with UV light (365 nm), the solution was transitioned from the free-flowing state to the gel phase. By using cross-sectional SEM images (Figure 2), the interior morphology of GelMA/PEtOx hydrogel was identified. These images show a three-dimensional porous structure that was intricately interwoven with pores that were typically 90  $\mu$ m in size. By loading of PEtOx into the system, a notable augmentation in pore size was observed in contrast to that of the pure GelMA hydrogel, which exhibited an average pore size of  $89.26 \pm 12.53$ . The outcome of this augmentation was an average pore size of  $92.24 \pm 2.61$ . When PEtOx was added to the reaction system, the wall thickness of hydrogel dramatically increased and improved its mechanical properties (as the wall thickness increased, the mechanical characteristics of the hydrogels improved [14]). This led to an augmentation of the mean pore diameter in relation to the volume unit, whilst concurrently producing a diminution in pore. These findings demonstrated that the amount of PEtOx synthetic polymer in GelMA/PEtOx hydrogel changes their average pore size, and as a result, changes the mechanical properties of the scaffold.

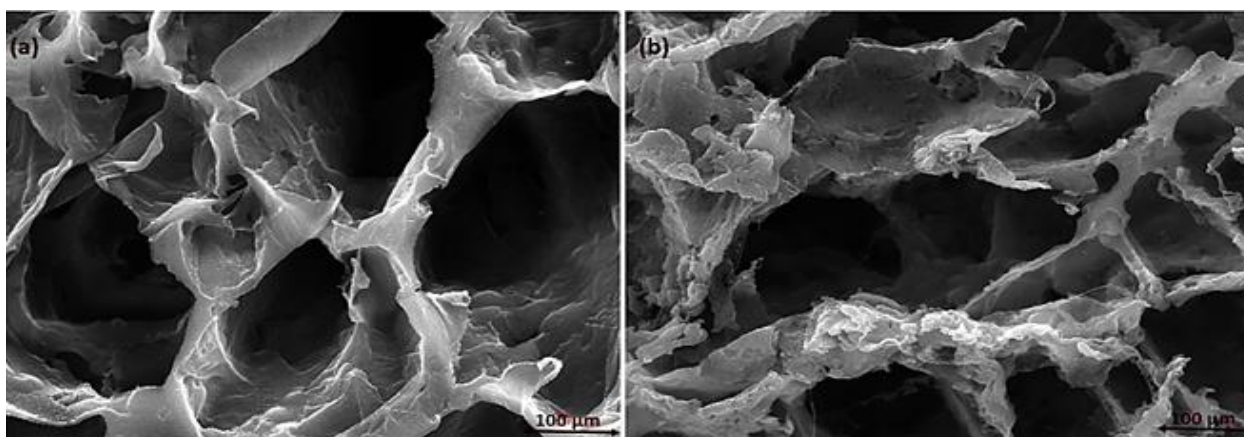


Fig 2 The Average Pore Size of Hydrogel in Cross-Sectional SEM Images (a) GelMA Hydrogel and (b) GelMA/PEtOx Hydrogel

### ➤ Infrared Analysis

Figure 3 displays the FT-IR spectrum of the sample of The GelMA/PETox hydrogel. The absorption band typically observed around  $3400\text{ cm}^{-1}$  is attributed to the stretching vibrations of O-H and N-H. The peaks in the range of  $2930\text{ cm}^{-1}$  and  $2400\text{ cm}^{-1}$  are ascribed to the stretching vibrations of C-H and O-H groups, respectively. The backbone structure of The GelMA/PETox hydrogel is linked to the absorption bands at  $1640\text{ cm}^{-1}$  (C=O stretching, amide I),  $1550\text{ cm}^{-1}$  (N-H bending, amide II and N-H bending,

amine),  $1460\text{ cm}^{-1}$  (CH<sub>2</sub> bending, alkane),  $1253\text{ cm}^{-1}$  (C-N stretching and N-H bending, amide III),  $1167\text{ cm}^{-1}$  and  $1074\text{ cm}^{-1}$  (C-N stretching, amine) [33]. It is noteworthy that the peaks at around  $570\text{--}670\text{ cm}^{-1}$  in the GelMA/PETox spectrum relate to the C-H bending of aromatic rings in PETox's chemical structure. Based on the FTIR spectra, it can be concluded that the GelMA/PETox hydrogel contains abundant amino and amide groups, which also confirms their high hydrophilicity.

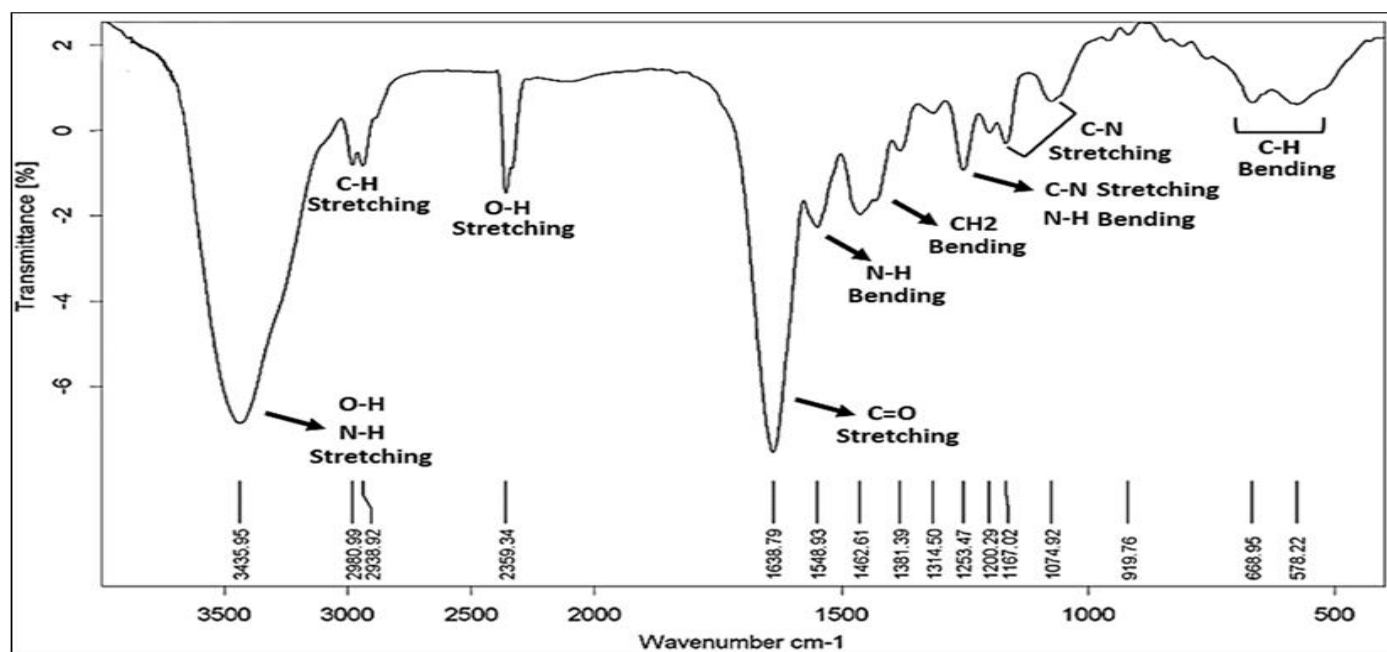


Fig 3 FTIR Spectra of the GelMA/PETox Hydrogel

### ➤ Swelling Ratio

By subjecting the freeze-dried GelMA/PETox hydrogels in PBS to a duration of 24 hours to achieve equilibrium at a temperature of  $37^{\circ}\text{C}$ , an analysis of the swelling ratio was conducted. Due to the high porous three-dimensional network structure of the hydrogel, it readily absorbs water when submerged in liquids. When the hydrogel dried, it absorbed 8 to 10 times its own weight in water. The interactions of the GelMA/PETox chains with water molecules caused the polymer to swell, but the cross-linking did not compromise the integrity of the polymer. Weighing the hydrogel every 60 minutes for ten hours allowed us to determine how quickly it swelled. GelMA/PETox hydrogel had a lower swelling ratio than GelMA hydrogel. The volume of GelMA/PETox hydrogel and GelMA hydrogel increased during the first three hours and then stabilized after five hours of immersion in PBS, as shown in Figure 4.a. The volume of GelMA/PETox hydrogel remained substantially steady from the sixth hours, and then the partial degradation of the hydrogel began, which is crucial. The poor swelling ratio of the GelMA/PETox hydrogel can be attributed to two factors. Hydrogen bonds between the gelatin strands initially prevented the swelling of the hydrogel. Second, adding PETox to gelatin made the gelatin network more restricted and the covalent cross-linking density between the PETox chains and GelMA chains increased [34]. The hydrogel is

excellent for implantation in low-pressure areas due to the high degree of cross-linking, which also improves the rigidity of hydrogel and reduces the swelling ratio. To strengthen the swelling qualities, the synthetic polymer PETox must be present in addition to the natural polymer GelMA. Therefore, an increase in concentration of PETox amplifies the rigidity of hydrogel and the extent of cross-linking, resulting in a rapid equilibrium time for swelling and a diminutive swelling ratio.

### ➤ Degradation Rate (Weigh Loss Analysis)

Due to the lengthy duration required for the regeneration of articular cartilage, and the advantageous gradual growth of new tissue during repair facilitated by the slow degradation rate of hydrogel, it is imperative that hydrogels possess a slow degradation rate when employed as a cell delivery system in cartilage tissue engineering [14]. Figure 4.b and 4.c displays the weight reduction outcomes of the created hydrogels. The findings indicate that while the degradability of GelMA/PETox hydrogel slowed down and the amount of weight loss decreased, the degradability of this group is still suitable. It is believed that the hydrogel scaffold entirely dissolved by the end of the fifth week following the injection time because the percentage weight loss simultaneously with time indicates about 43% weight loss within two weeks, indicating that the rate of biodegradation has increased with time (Figure 4.d).

Overall, the hydrogels lost a significant amount of weight, which supports the claim that the GelMA/PEtOx hydrogel is biodegradable. Two factors were identified as contributing to the degradation rates of the GelMA/PEtOx hydrogel. First, the enzymatic breakdown of gelatin by the RGD sequence. Second, the pore diameter of GelMA/PEtOx

hydrogel increased over time, thus changing the pace and volume of water absorption and speeding up the disintegration process. The slow degradation rate of GelMA/PEtOx hydrogel in the least possible time made it suitable for long-term application in the human body [34].

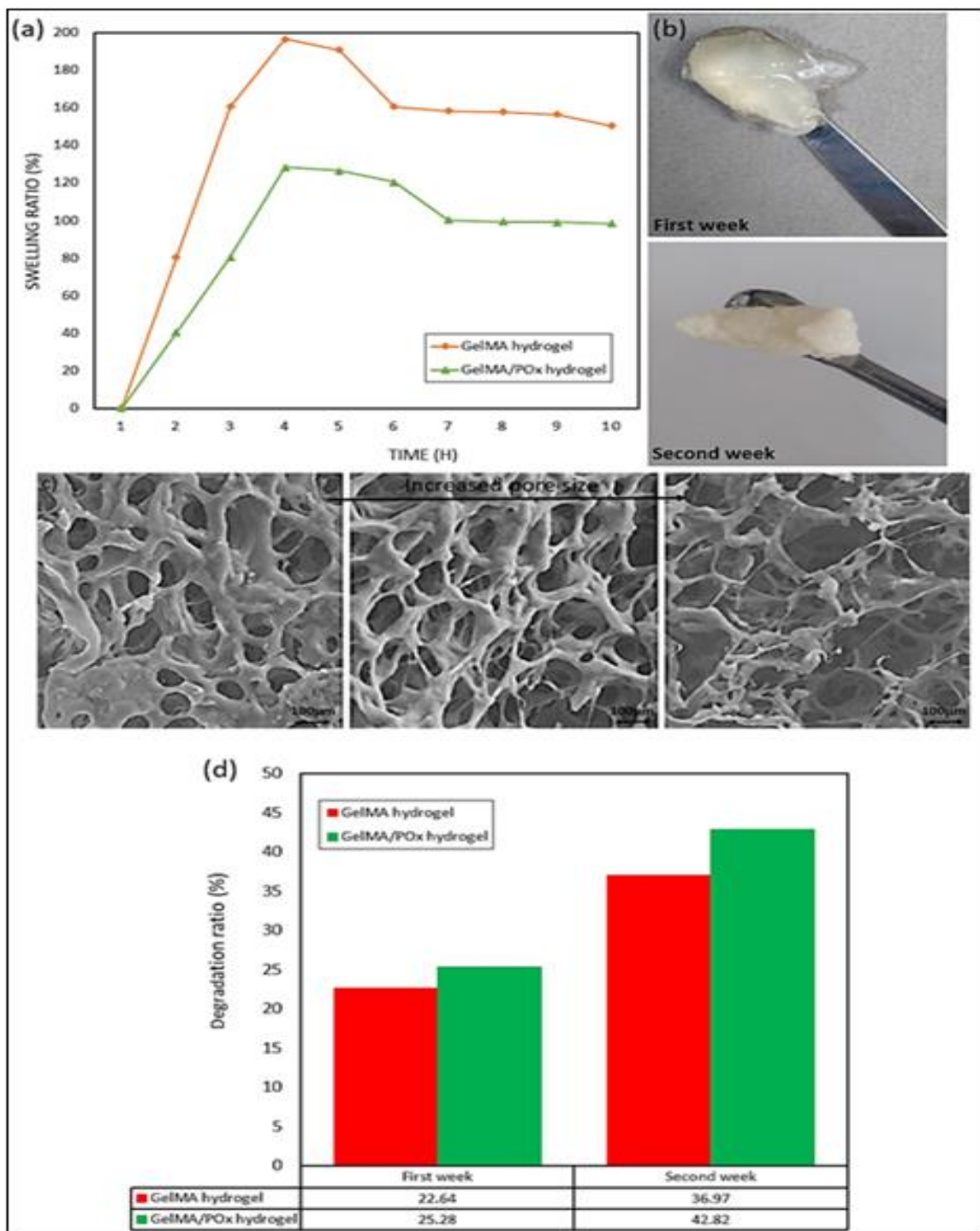


Fig 4 Swelling and weight loss analysis, (a) chart of swelling ratio of GelMA/PEtOx hydrogel for 10 hours, (b) degradation percentage of GelMA/PEtOx hydrogel in the first and second weeks, (c) In the first week, the SEM image shows the GelMA/PEtOx hydrogel with a relatively low degradation percentage, which is sticky, elastic and quite flexible. Over time, the SEM image of the damaged GelMA/PEtOx hydrogel in the second week shows that its structure has become completely dry and fragile. As shown, the pore sizes have grown and the quantity of damage has increased, (d) The assessment of the analysis of weight reduction pertaining to GelMA and GelMA/PEtOx hydrogels post 1 and 2 weeks is hereby presented.

➤ *Porous Structures*

For a hydrogel scaffold, the porosity characteristics of a material are essential because they promote cell adhesion, penetration, proliferation, and exchange of nutrients and oxygen [20]. The behavior and viability of cells are significantly influenced by the porosity and pore size of polymeric materials. In the present study, the morphology of the frozen material was investigated employing a scanning electron microscope. In Figure 2, the SEM micrograph is displayed. The porous interior structure of hydrogels was visible in the electron micrographs. When ice crystals disrupt the hydrogel structure during the freeze-drying process, this structure is what is left behind. Pore size reduces, and interporous area rises as the crosslinker fraction rises. Higher swelling ratio and lower mechanical strength are caused by increased porosity. In comparing the GelMA/PEtOx hydrogel to the GelMA hydrogel, this figure shows a drop in the number of pores but an increase in the size of the pores. When the free water content is compared to the morphology of the hydrogels, the reduction in free water content with increasing cross-linking percentage may be explained by the smaller pores of hydrogels [35]. According to this test, the addition of PEtOx raised the porosity of hydrogel from  $87.6 \pm 14.4$  to around  $91.5 \pm 3.8\%$ , demonstrating the three-dimensional structure of hydrogel. PEtOx concentration seems to affect the porosity of GelMA/PEtOx hydrogel.

➤ *Investigating the Hydrophilicity and Hydrophobicity of the GelMA/PEtOx Hydrogel*

By absorbing a lot of water, the hydrogel matrix can mimic human body tissue since human tissues contain a lot of water (between 65 and 80% of cartilage tissue is made up of water). This property confirms the hydrophilicity of the hydrogel matrix. As a consequence, cellular adhesion and the process of wound healing transpire at the location of injury owing to the hydrophilic nature of the hydrogel. Before that, using infrared analysis, we found that the synthesized hydrogel has various amide and amine groups, which can contribute to the hydrophilicity of the hydrogel. Besides that, a technique for determining whether a surface is hydrophilic or hydrophobic is to measure the water contact angle with the surface. Based on this test, a  $2\mu\text{L}$  drop of water was gently put on the surface of the hydrogel, and the time it took for the drop to make contact with the hydrogel was measured. This technique involves placing the droplet on the substrate and then gradually tilting it. Just as the droplet begins to move, the leading contact angle in front of the droplet is calculated. At the same time, the retreating contact angle of the droplet is measured. If a water droplet forms a contact angle of less than  $90^\circ$ , the surface is deemed hydrophilic. Conversely, if the contact angle is greater than  $90^\circ$ , the surface is considered hydrophobic, causing water droplets to form beads and attach to each other more than to the surface itself. In the event that the effective contact angle of water drops exceeds  $150^\circ$ , the material is identified as superhydrophobic, also referred to as ultrahydrophobic (Figure 5.a) [36]. Figure 5.b illustrates the contact angle of GelMA/PEtOx hydrogel matrix with water, which is  $43.4 \pm 2.2$  degrees, confirming the hydrophilicity of GelMA/PEtOx hydrogel matrix.

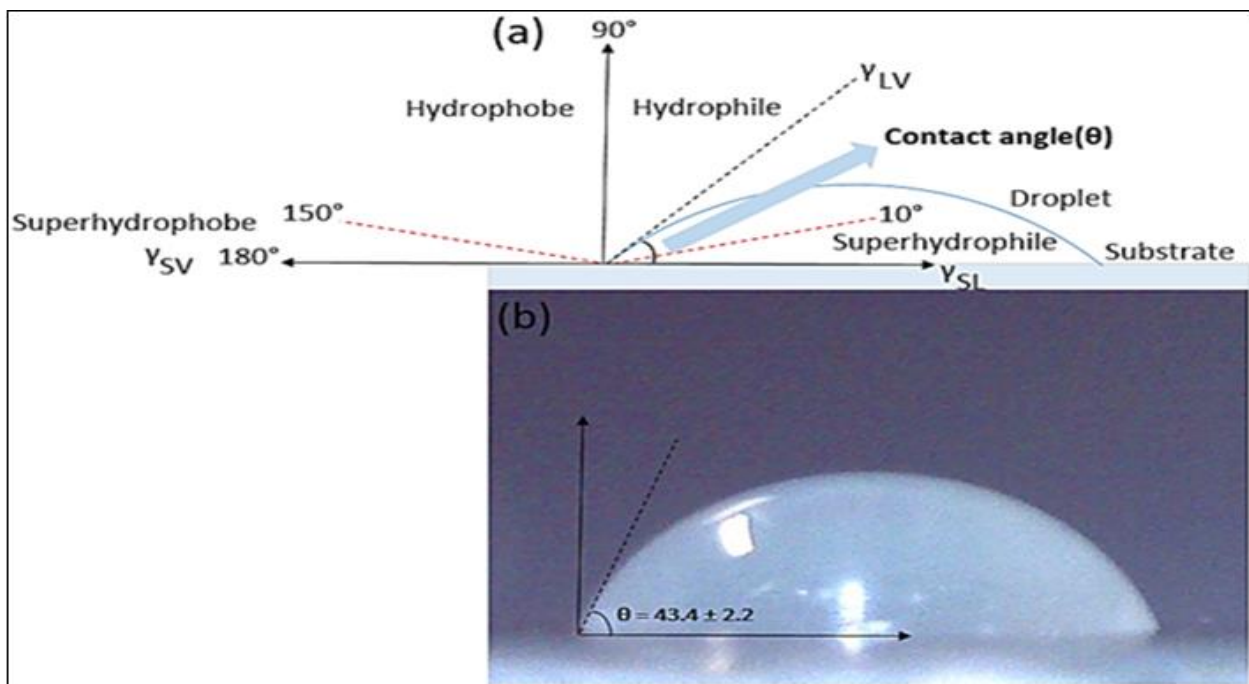


Fig 5 Investigating the hydrophilicity of hydrogel, (a) Contact angle as a quantity of wettability (contact angle ( $\theta$ ) of a droplet is typically measured from a horizontal vantage dot at the point where the droplet surface and substrate come into contact. It is described as the angle formed by the tangent droplets on the surface and the substrate surface [37]), (b) contact angle of a water droplet with the GelMA/PEtOx hydrogel.



➤ *Elastic Modulus for the GelMA/PETox Hydrogel*

Numerous factors affect how articular cartilage behaves mechanically. The creation of tissue with varying qualities in the depth direction is caused by the heterogeneous distribution of proteoglycans and collagen fibers in the direction of the thickness of the articular cartilage. These characteristics directly affect how much stress, strain, and shape change the cartilage. From a mechanical standpoint, articular cartilage is a heterogeneous substance, and the type of dispersion and orientation of collagen fibers, which is different in each density of layer and direction, affect the behavior of the material in two compression and tension states that are the most crucial components of this behavior. This indicates whereas the non-fibrous section of the tissue bears the majority of the stresses in compression, the collagen fibers take the majority of the forces during stretching (consisting of synovial fluid and proteoglycan). As a result, articular cartilage exhibits two distinct behaviors in compression and tension. These behaviors are referred to as viscoelastic behavior, and they occur in similar ways in many other soft materials. Because of this, they replicate the behavior of differentiating hydrogels to articular cartilage, which should be within the range of the body's natural cartilage [38, 39]. Here,

stress/strain testing was used to examine the elastic properties of GelMA/PETox hydrogel and the imaging was recorded from the moment the hydrogel was stretched until it broke for almost one minute (Figure 6.a). The elastic modulus was calculated using stress-strain curves, as shown in Figure 6.b. For 10% GelMA and 35% PETox, 10% GelMA and 45% PETox, 20% GelMA and 35% PETox, and 20% GelMA and 45% PETox hydrogels, respectively, the elastic modulus was  $390.24 \pm 0.21$  kPa,  $402.43 \pm 0.15$  kPa,  $573.17 \pm 0.23$  kPa, and  $707.31 \pm 0.11$  kPa. Cartilage has an elastic modulus of 500-900 kPa. As anticipated, the increased GelMA/PETox hydrogel content of sample 4 is closer to the elasticity modulus of cartilage tissue. The behavior of stress-strain and the elastic modulus of GelMA/PETox hydrogel were significantly affected by increasing PETox concentration. This resulted from the more enormous walls of hydrogel and increased cross-linking level. Based on simulation of articular cartilage that also occurred in these samples, the stress grows exponentially with increasing strain since the properties of cartilage are not precisely the same from the outside to the inside. We selected GelMA 20% and PETox 45% hydrogel as the experimental group in cellular studies based on its elastic property criteria.

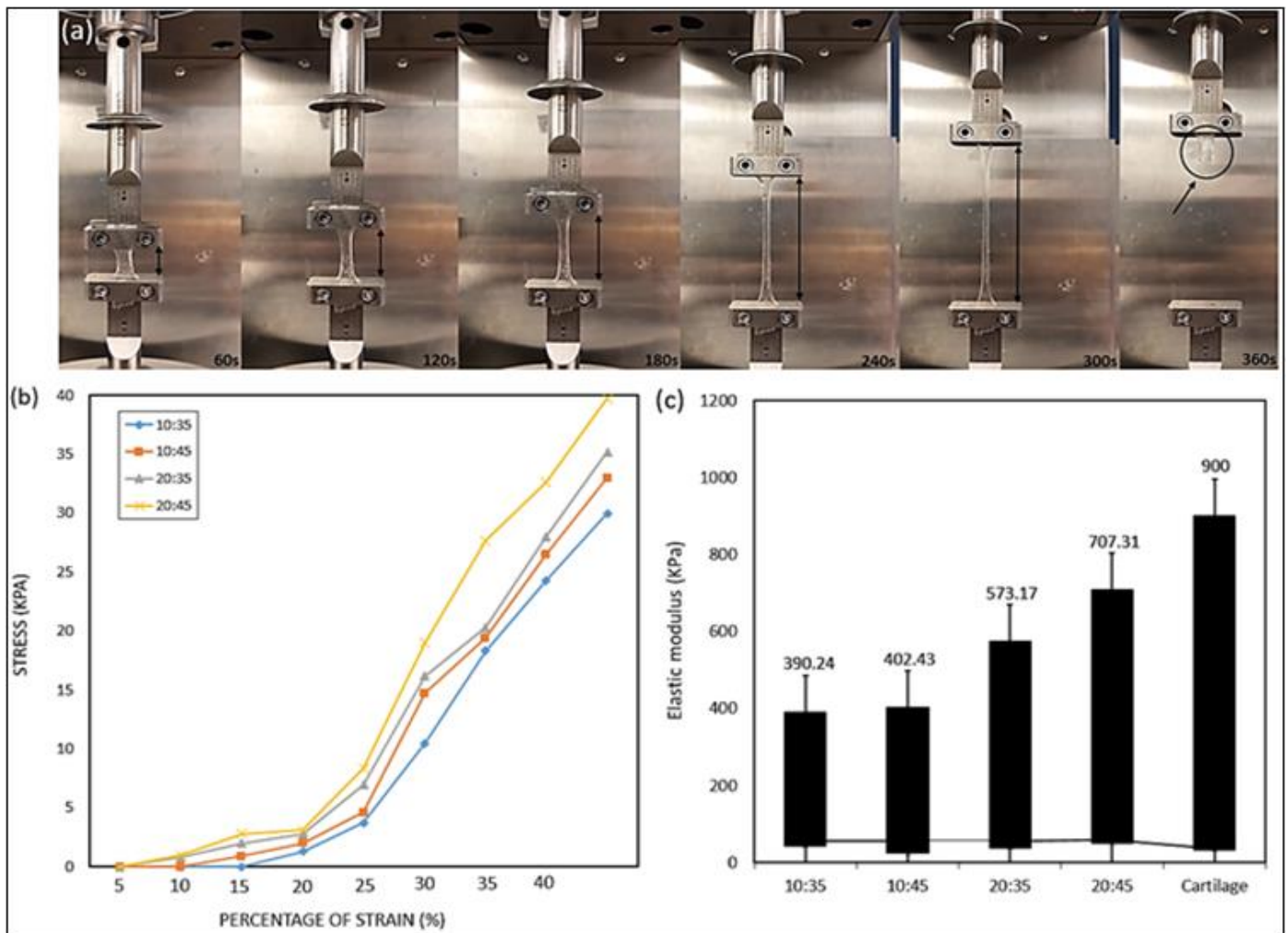


Fig 6 Examining the modulus of elasticity of hydrogel (a) Pictures taken from the moment of stretching until breaking the hydrogel (approximately one minute). (b) Stress-strain curves of samples of GelMA/PETox hydrogel. (c) comparison of elastic modulus of samples with articular cartilage.

### ➤ Blood Compatibility

Hemolysis denotes the process of disintegration or rupture of the red blood cell membrane, leading to the liberation of hemoglobin into the blood plasma, resulting in the plasma acquiring a red and lucid appearance. Hemolysis may be done under the influence of various factors, such as osmotic pressure, chemicals such as alcohol, poisons, and hemolysis, and incompatible injection, and if a biomaterial is used, it occurs if it is incompatible with a blood tissue of the body [40]. Hemolysis is characterized by premature destruction of red blood cells. Therefore, a primary attribute

that must be taken into account is the blood compatibility of biomaterials. Upon conducting an analysis of the hemolytic rate of the hydrogels that had been prepared, it was duly observed that the hemolysis of the GelMA/PEtOx hydrogel amounted to  $0.83 \pm 0.06$ , whereas that of the GelMA hydrogel was  $0.67 \pm 0.02$ . It is noteworthy that both values were significantly lower than the positive control, which was measured at  $2.20 \pm 0.04$ . This observation further corroborates the fact that the aforementioned hydrogels possess blood compatibility.

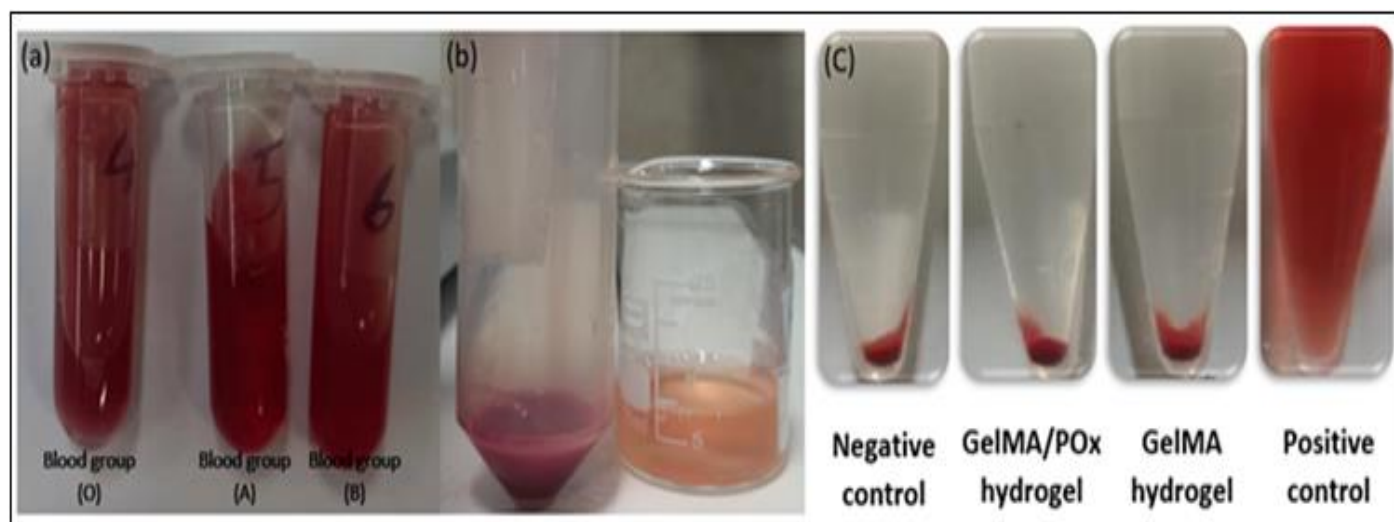


Fig 7 Blood compatibility analysis, (a) Different blood groups of donors for compatibility blood test, (b) Separation of plasma from blood, (c) Pictures from the hemolytic activity test of the hydrogels in four groups, negative control, GelMA/PEtOx hydrogel, GelMA hydrogel and positive control.

### ➤ Drug Release Analysis

The in-vitro release index of KGN from the GelMA/PEtOx hydrogel was assessed at varying concentrations to determine the ability of the GelMA/PEtOx hydrogel to release the KGN drug at the observed proportion. UV-visible spectroscopy was employed to assess the liberation of KGN from the GelMA/PEtOx hydrogel. The hydrogels were immersed in simulated body fluid (PBS) with a pH value of 7.4 at a temperature of 37°C. At different times, the hydrogel supernatant was removed and centrifuged, and to determine the release rate of KGN, the absorbance of the removed liquid was recorded at 285 nm [41]. First, to draw the absorption standard curve of KGN in PBS, different concentrations of 0, 20, 40, 60, and 80% of this substance were prepared (Figure 6.a and 6.b) and by obtaining the absorption rate, it was drawn the absorption standard curve (Figure 6.c). Then, utilizing the graph that was acquired, an analysis has been conducted on the KGN release profile from the GelMA/PEtOx hydrogel in varying concentrations (2, 6, and 10  $\mu\text{g}/\mu\text{l}$ ). The purpose of this evaluation is to show the ability of the prepared hydrogel in

the controlled release of related clinical is KGN. Figure 6.d shows the cumulative amounts of KGN released over two weeks. KGN loaded at the microgram level exhibited a large release on the first day followed by a sustained rate-controlled release for up to 10 days for all loading amounts of KGN. As per the data presented in Figure 6.d, it can be inferred that the quantity of drug release for the 10  $\mu\text{g}/\mu\text{l}$  dosage of KGN is remarkably elevated until the fourth day, which can elicit an inflammatory response and result in toxicity. Furthermore, owing to the meager quantity of KGN at a 2  $\mu\text{g}/\mu\text{l}$  dose, it is imperative to study the impact of the drug KGN at a 6  $\mu\text{g}/\mu\text{l}$  dose to continue with the cytotoxicity assessments. Also, Figure 6.e shows the difference in the release of KGN in GelMA hydrogel and GelMA/PEtOx hydrogel at a concentration of 6  $\mu\text{g}/\mu\text{l}$ . According to this figure, more than 90% of the drug has been released in the hydrogel without PEtOx on the first day. However, the presence of PEtOx in the scaffold network controls the release rate and will cause continuous release of the drug in the cartilage tissue damage environment.

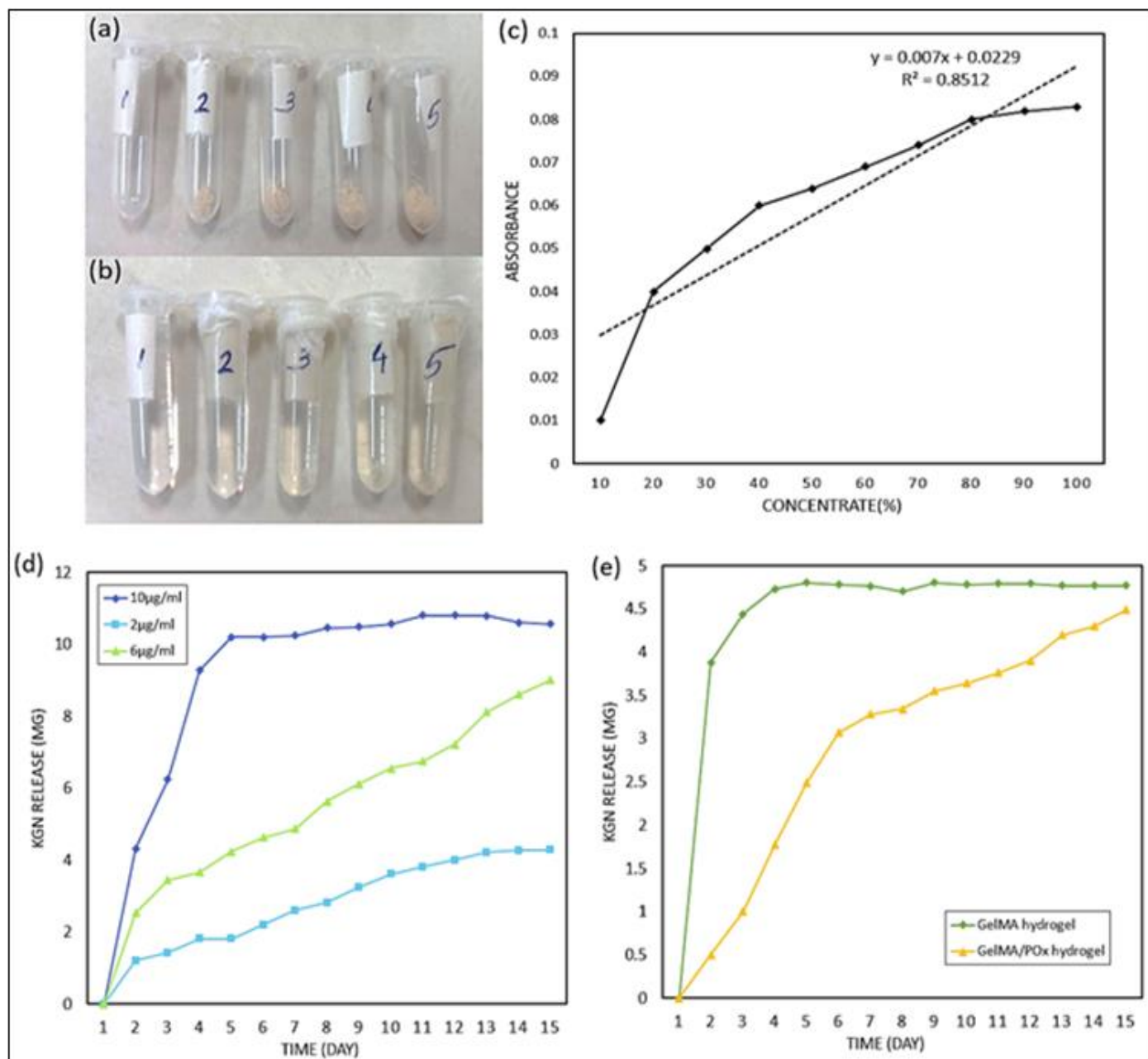


Fig 8 The in-vitro release of KGN from GelMA/PEtOx hydrogel in PBS with pH 7.4 at 37°C. (a) KGN at different concentrations %0, %20, %40, %60 and %80 (b) Different concentrations of KGN were dissolved in PBS (c) Beer-Lambert diagram for KGN in PBS solution (d) Release of KGN in GelMA/PEtOx hydrogel at different concentrations 2,6 and 10 µg/ml (e) KGN release from GelMA and GelMA/PEtOx hydrogels at a concentration of 6 µg/µL

➤ *Viability/Cytotoxicity Assay*

NIH/3T3 cells were sown in the scaffold to evaluate the biocompatibility and toxicity rate of the KGN medication. By using assays for cell viability, cell proliferation, and cell adhesion, the biocompatibility of the scaffold was assessed. Live/dead staining was used to measure cell viability. The percentages of living cells in the

presence of KGN drug loaded within GelMA/PEtOx hydrogel in a cell culture medium and the saline control media were calculated from the flow cytometry analysis, and they were 74.995% and 75.019%, respectively. The culture of GelMA/PEtOx hydrogel without KGN drug was 74.997% (Figure 7).

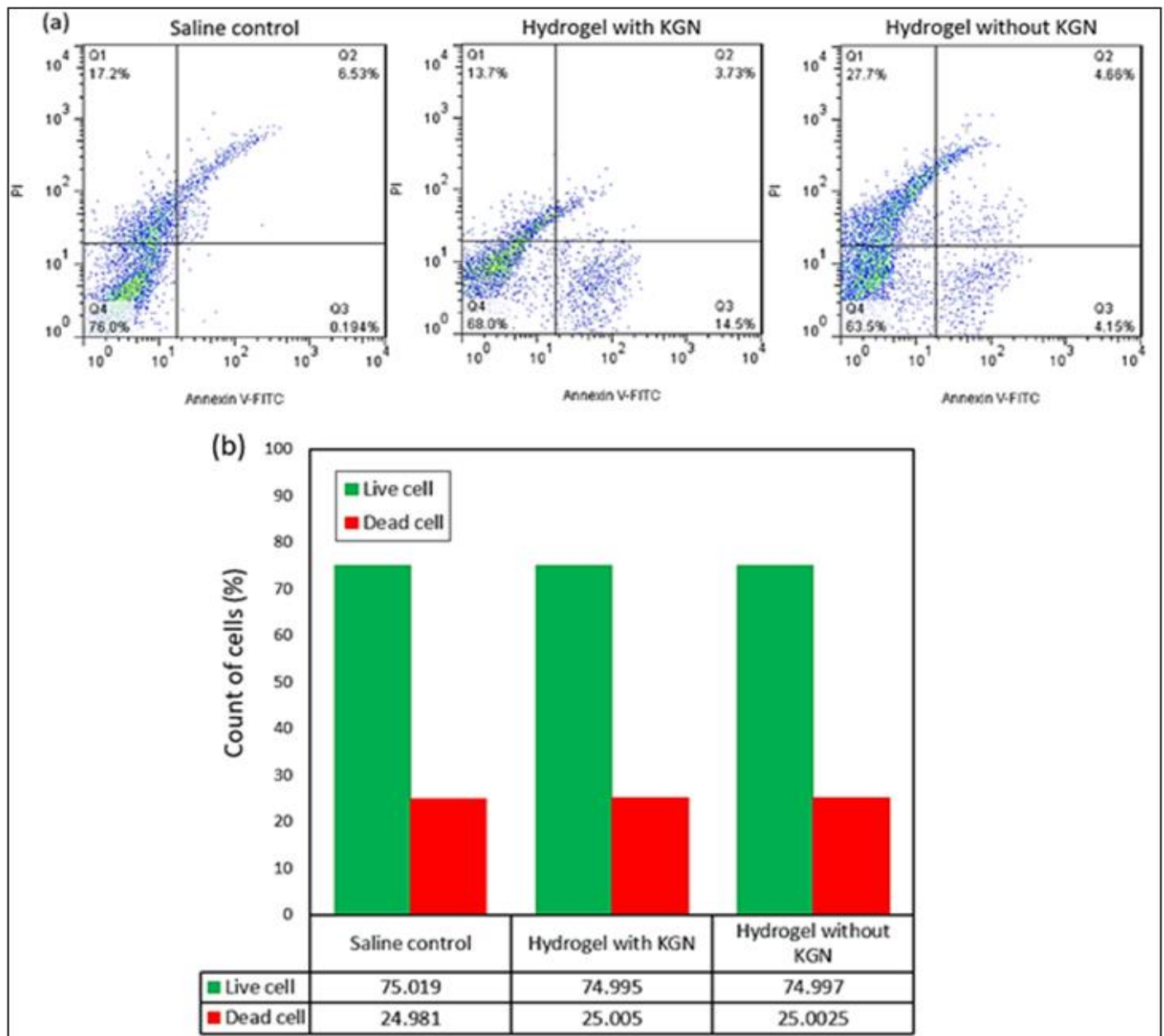
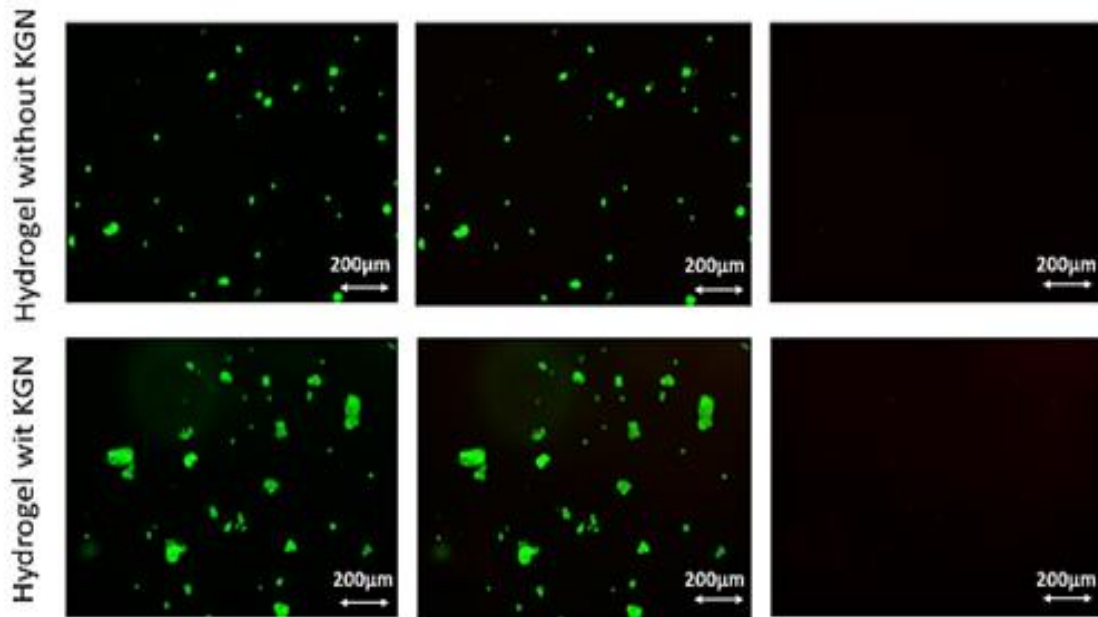


Fig 9 Cell viability analysis, (a) Flow cytometry test results in the saline control medium, hydrogel medium with KGN drug, and hydrogel environment without KGN drug, (b) the percentage of cell survival and death in the culture medium: saline control, hydrogel with drug and hydrogel without drug.

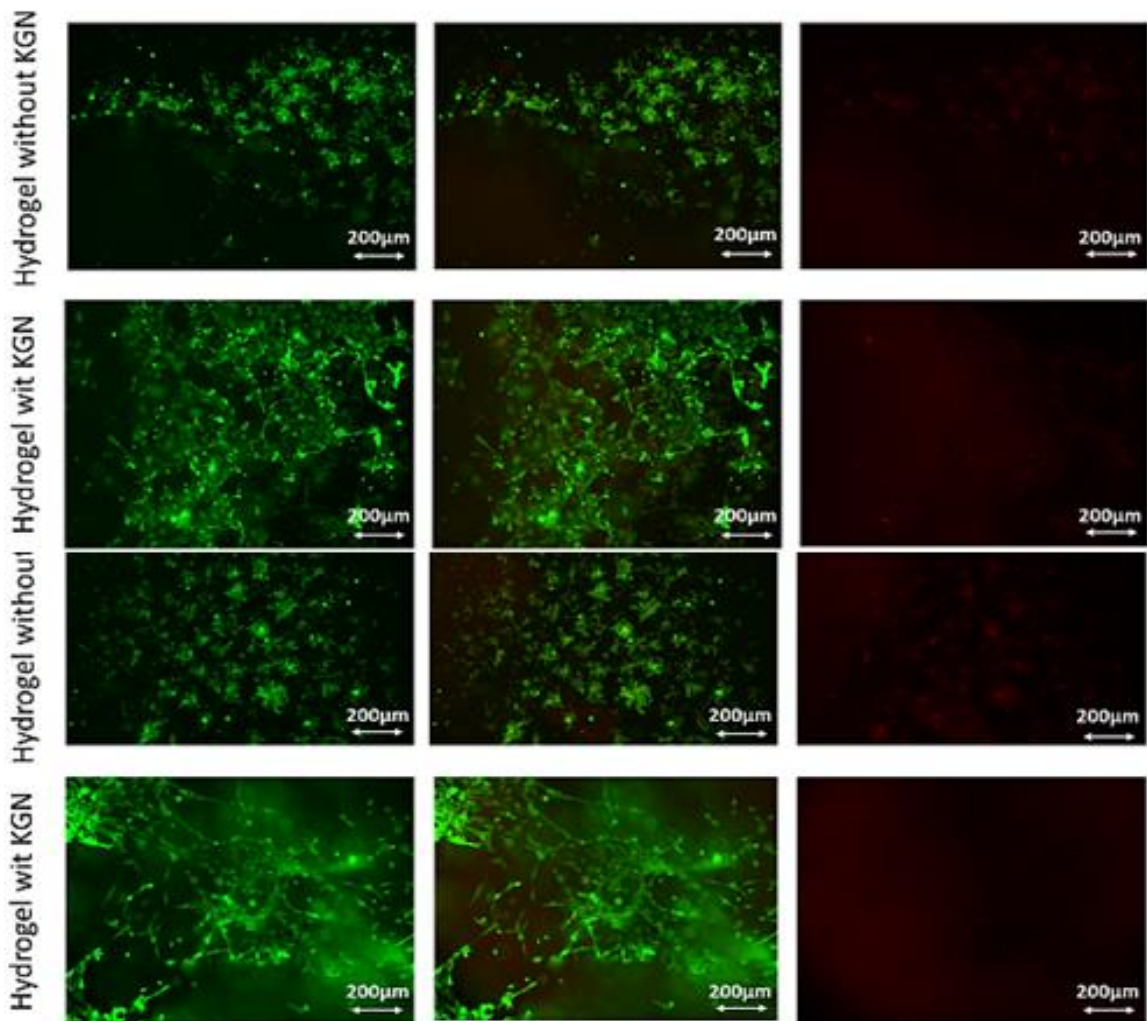
As can be shown, a significant portion of the cartilage tissue fibroblast cells was able to survive in the synthetic hydrogel culture medium, indicating that the KGN drug-containing scaffold had a high degree of adhesion. Due to the perfect safety of KGN and its exceptionally low cytotoxicity, the percentage of cell survival in the environment where the hydrogel does not contain medicines is approximately %0.002 greater than in the environment where the hydrogel and the drug are combined [42]. Figure 10 displays the cell adhesion on the first, third, and seventh days in the presence and absence of KGN medication by switching the channel from a single point and using a confocal microscope. In the medium containing KGN drug,

a perfect state of connection, adhesion, and network can be seen, which proves that the cells, due to the presence of KGN drug, in addition to a high percentage can survive, and can easily stick to the constructed scaffold and provide the possibility of cell transfer and proliferation inside the hydrogel, which in turn will eventually result in the repair and improvement of the desired cartilage tissue by the end of the fifth week. These findings confirmed the ability of GelMA/PETox hydrogel containing KGN to increase cell adhesion by preserving cell population and exhibiting minimal cytotoxicity, which may be advantageous for usage in cartilage regeneration following in-vivo implantation.

(a) Day 1



Day 3



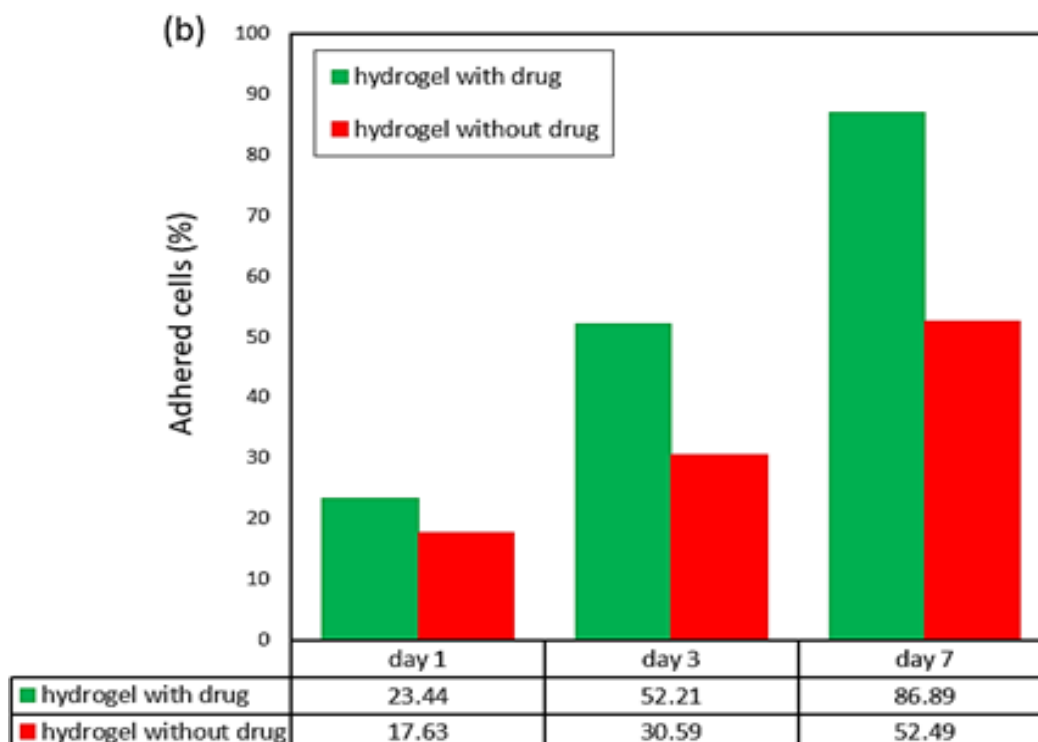


Fig 10 Analysis of cell adhesion and uptake in-vitro, (a) Representative fluorescence images, ascertained through Live/Dead staining assay, have been rendered to display the viability of chondrocytes co-cultured for the purpose of examining cell adhesion in the presence and absence of KGN drug with the GelMA/PEtOx hydrogel over the course of 1, 3 and 7 days. (b) Quantitative evaluation of adhered NIH/3T3 fibroblast cells.

#### IV. CONCLUSIONS

In the present investigation, a newly developed hydrogel, GelMA/PEtOx, which is both biocompatible and biodegradable, was synthesized through the process of UV photo-crosslinking. This hydrogel also contains KGN medication. The physicochemical characteristics of the hydrogels, including morphology, swelling ratio, degree of hydrophilicity, degradation rate, porosity, mechanical features, blood compatibility, drug release, cell culture, and cell survival, were examined. The outcomes showed that the GelMA/PEtOx hydrophilic hydrogel displays partly high of swelling ratio and degradation percentage. We found in SEM analysis that the GelMA/PEtOx hydrogel has porous structure with uniform pore size. The strength of the wall of this porous structure can be increased by increasing the concentration of synthetic polymer PEtOx which results in an increase in the elastic modulus of this hydrogel. The experiment concerning hemolysis has revealed that the hemolysis rate of GelMA/PEtOx hydrogel was found to be  $0.83 \pm 0.06$ . Furthermore, the blood compatibility performance was deemed to be of excellent quality. Besides, KGN drug release rate from the hydrogel was quite stable during two weeks. Cell viability analysis of encapsulated NIH/3T3 cells in the hydrogel containing KGN also showed that cytocompatibility for adherence and cell proliferation in GelMA/PEtOx hydrogel containing KGN was perfectly suitable. These results show that GelMA/PEtOx hydrogel paves the way for future hydrogel implant or inject in cartilage damaged tissue with loading stem cells, drugs cartimaterials for minimal host response.

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