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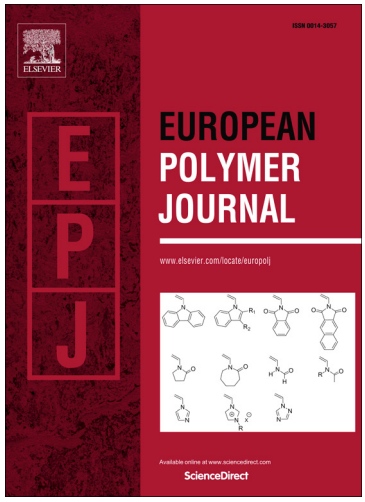

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Thermal and kinetic evaluation of biodegradable thermo-sensitive gelatin/poly(ethylene glycol) diamine crosslinked citric acid hydrogels for controlled release of tramadol

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Keywords: Hydrogel, drug release, tramadol, biocompatible, biodegradable

Abstract

Nowadays, hydrogels have become ideal materials for use in biomedical applications by virtue of their biodegradability and biocompatibility. In this study, poly(ethylene glycol) diamine (PEGD) based hydrogels were synthetized using as crosslinking agents citric acid (CA) or glutaraldehyde (GTA), and gelatin (GEL) as hydrogel vehicle. The hydrogels were studied for drug release in vitro using tramadol (TR) as a model drug. Thermal studies under isothermal and non-isothermal conditions were conducted. The elastic module (G') showed higher values than the loss module (G'') confirming that the contribution of the elastic segments in both materials is more significant than the viscous ones. Aqueous stability, swelling and drug release properties were determined. The swelling analysis indicated that both hydrogels are temperature dependent. The kinetics studies showed an anomalous drug release mechanism. PEGD:CA/GEL hydrogel behave as an elastic matrix strong enough to maintain the drug dosage.

1. Introduction

Crosslinked polymers exhibit excellent biocompatibility properties and have been used in the biomedical and pharmaceutical areas [1,2] mainly due to their good interaction with living tissues [3]. One type of these three-dimensional (3D) networks that show high ability to absorb large quantities of water while remaining insoluble in aqueous solutions [4,5] are the hydrogels, also called "smarts materials" due to their capability to respond to external stimuli either physical, chemical, and/or mechanical. This interesting feature allows the hydrogels have a certain self-control of the mechanisms involved in their response [6]. For example, a change in the system environment (pH, temperature, ionic strength, etc.), can promote a phase transition or increase its volume and hence, change the size of the internal pores in the polymer matrix [7]. The hydrogels ability to swell and retain a significant portion of water when placed in an aqueous or biological solutions is produced by the presence of hydrophilic functional groups, which allow them to form soft and elastic materials with physical, chemical and biological properties that can be manipulated through their parameters of synthesis [8]. The correct performance of the hydrogels depends strongly on their properties and chemical functionality, so a suitable selection of polymer microstructure is extremely important taking into account the required application [9-11]. Although, natural polymers are more suitable for biomedical applications due to their high biodegradability, biocompatibility and biologically recognizable moieties that support cellular activities [12] have the disadvantage of poor mechanical properties.

Poly(ethylene glycol diamine) (PEGD) is a biocompatible, biodegradable and non-toxic biopolymer which resists recognition by the immune system [13]. PEGD can react with a

crosslinking agent forming a three-dimensional mesh, which is often used as vehicle for the development of controlled drug release systems [13]. Some studies carried out with PEGD/genipin scaffolds indicated that they are excellent biomaterials for the cartilage regeneration [14]. In addition, PEG-4D (tetra-amine) has been used for tissue regeneration with good results [15]. Moreover, because the PEGD is a nontoxic nonionic surfactant, has been used in formulations for parenteral applications [16]. In this regard, Miljkovic et al. [17] synthesized and characterized a novel biocompatible and biodegradable hydrogel comprised of PEGD/genipin/gelatin that can be used as an injectable hydrogel for cartilage repair. In this 3D system, the gelatin provides a viscoelastic consistency to the template, allowing it to be injected, and the genipin acts as crosslinking agent. The results of this research showed that if the synthesis parameters are handled, the hydrogel characteristics could be changed in terms of degradation times and mechanical properties.

Crosslinking agents in such applications play a very important role because they allow the formation of the connections in the polymer to obtain the three-dimensional mesh [11]. Some of the crosslinking agents most commonly used for biomedical purposes are dextran [17-19] , glutaraldehyde (GTA) [20-22] and genipin .[16,23,24] However the first two have certain toxicity, and the genipin (which is 10,000 times less toxic than GTA [25]) has the inconvenience of being a high cost material, hence, it is not affordable for pharmaceutical applications.

In this work, we offer a new alternative by using citric acid (CA) as crosslinking agent to generate new low-cost 3D polymeric scaffolds. We chose CA because is a renewable resource-based substance, mainly manufactured by the fermentation of carbohydrate, starch or glucose [18]. CA has a poly functional nontoxic nature because is also produced as

metabolic product of the body (Krebs or citric acid cycle) in all living cells that use oxygen as part of the cellular respiration. In addition, CA has excellent properties such as high cytocompatibility and solubility [18,19] and is commercially available at low cost.

PEGD:CA 3D network as vehicle for drug controlled release was evaluated by using tramadol hydrochloride (TR) as therapeutic drug model. TR is a central acting analgesic having both opioid and nonopioid effects. It is administered when non-steroidal anti-inflammatory drugs fail to mitigate pain [20-27]. The half-life of the drug is about 6.3 hours and the usual oral dosage regimen is 50 to 100 mg every 4 to 6 hours with a maximum dosage of 400 mg/day [28]. Therefore to reduce the frequency of administration and to improve the patient compliance, a sustained-release formulation of tramadol is desirable. The main advantages of using PEGD:CA scaffolds as vehicles for the controlled release of TR is the high biodegradability and biocompatibility that this polymeric matrix can offer when is in contact with biological fluids, and the facility with which their physicochemical properties can be modified. Thus, by selecting the proper parameters of synthesis, the properties of PEGD:CA 3D network can be tuned for use it as vehicle for drug delivery or as template for tissue engineering, cell growth or to encapsulate antibodies for the development of new generation of vaccines (this last is under investigation in our research group).

One difficulty that has to be overcome during the design of TR controlled release system is the high solubility of this medicament in water. In this regard S. Tiwari et.al. [24] reported the effect of hydrophilic (hydroxypropyl methylcellulose [HPMC]) and hydrophobic (hydrogenated castor oil [HCO], ethylcellulose) polymers on the release rate of tramadol [24]. In this work, the hydrophobic polymeric tablets resulted in a better sustained in vitro

drug release (>20 hours, 90% TR) when compared with the hydrophilic polymeric tablets (<14 hours, 90% TR). Meanwhile Acosta et al. [26] reported that the encapsulation of TR in alginate-chitosan microcapsules produced a TR maximum release of 86 % at 24 h, this was evaluated in simulated intestinal fluid. Another example is the work of Naeem et al. [20] who developed TR-microparticles and TR-bilayer polymeric tablets, the maximum release of TR in these systems was of 99.7 and 92.6 % after 8 and 12 h respectively. It is important to notice that the maximum sustained release of TR in these systems was found at 24 h. Although these results are promising, further research has to be done to improve the biocompatibility and biodegradability of these polymeric vehicles, as well as to increase their performance during releasing for make it sustainable for longer period of time within the therapeutic concentration of the drug. Therefore, the main idea of this work is to contribute to the developing of low cost, biocompatible and biodegradable formulation that allow to reduce the frequency of the administration of TR while maintaining its analgesic effect.

2. Experimental Section

2.1 Materials

Gelatin (GEL) from porcine skin type A (powder bioreagent), poly(ethylene glycol) diamine (PEGD) (Mn=2000), citric acid (purity >98.5%), glutaraldehyde solution (grade I, 25% in H_2O) and tramadol hydrochloride (>98% HPLC), all these reagents were obtained from Sigma-Aldrich Co. (St. Louis, NO) and used as received.

2.2 Synthesis of Hydrogels

2.2.1 PEGD:Citric Acid/Gelatin (PEGD:CA/GEL)

The synthesis of PEGD:CA/GEL was performed in an environment free of biological contaminants (sterile atmosphere), this was needed because the interaction of the hydrogel with atmospheric pollutants promotes the growth of fungi. During the synthesis, three solutions were prepared and mixed according to the following procedure: solution 1 was obtained by dissolving 0.03 g of PEGD in 0.6 mL of deionized and sterile water (5% vol/wt%) the solution was maintained under stirring (200 rpm) at 60 °C. Solution 2 (0.05 M) was prepared by dissolving 0.0096 g of CA in 1 mL of deionized and sterile water (this was the crosslinking solution). Solution 3 was obtained by mixing 0.5 g of GEL in 2 mL of deionized and sterile water to obtain a 25 % vol/w suspension. Solutions 1 and 2 were mixed and vigorously stirred for 45 min, at this time the crosslinking reaction occurs between PEGD and CA, the reaction temperature was kept at 60 °C. Once completed the reaction time, the solution 3 was added to the mixture. The resulting suspension was stirred for 10 min (the quantities used of each reagent to obtain the different formulations studied in this work are reported in Table 1). After obtaining the hydrogel, a controlled cooling process was carried on at 1°C/min rate (60 °C to 25 °C). During this procedure, a bath cooled with recirculating (LabTech LCB-R08) was used. Finally, the hydrogels were washed and dried in a stove (SSI-DHG-9053A) at 45 °C for 48 hours.

Table 1. Hydrogel formulation.

EXPERIMENT (PEGD:AC)	**PEGD 5 wt/vol% (mL)**	**CA-GTA 0.05M (mL)**	**GELATIN 25 wt/vol% (mL)**	**TRAMADOL (mg)**
1:1	1	0.2	2	25
1:2	1	0.4	2	25

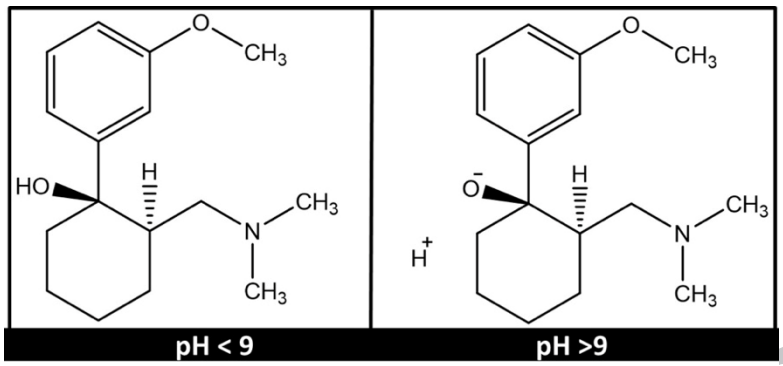
1:5	1	1	2	25

2.2.2 PEGD:Glutaraldehyde/Gelatin Hydrogel (PEGD:GTA/GEL)

The synthesis of the PEGD:GTA/GEL hydrogel was followed according to the procedure explained above, by substituting the CA for GTA, in this system the reaction time was 45 min. It is important to mention that during reaction the solution turned brown indicating the formation of the 3D hydrogel.

2.3 Composite

The drug-composite was obtained by slightly modifying the explained protocols. Briefly, in solution 1 and after the PEGD dissolution, 25 mg of tramadol (TR) were added, the resulting mixture was stirred for 15 min maintaining the temperature at 60 °C. At this reaction temperature TR remains stable (according to the TGA analysis) and it is assumed that does not react with any species during the composite formulation. Afterward, the solutions 2 and 3 were added following the procedure described before. The pH of the reaction media was maintained at 6.5, therefore, TR was captured inside the polymeric matrix in its non-ionized form as it is shown in the scheme 1 [29,30].



Scheme 1

2.4 In Vitro Drug Release Study

To evaluate the drug delivery, the concentration of TR released was measured at specific intervals of time. The experimental procedure was as follow: samples of 0.5 g of PEGD:CA/GEL/TR or PEGD:GTA/GEL/TR composites were placed inside of filter paper bags which served as hydrogel reservoir. These samples were placed separately in beakers filled with 40 mL of deionized water. Then, the delivery systems were placed in a batch system adapted with a recirculating water device, which kept the samples at the desired temperature (25°C or 37°C). During the experiments, the samples were stirred at 130 RPM to assure the homogeneity of the solution. The measurements of drug release were performed in a Cary 60 UV-Vis spectrometer by selecting the wavelength of the maximum absorbance of tramadol (271 nm). Then, a calibration curve in the range of 20 to 625 ppm was obtained (r^2=0.999). Aliquots of 1 mL were taken from the solutions at different intervals of time, afterward the absorbance were measured and the concentrations calculated with the following equation C=[A+0.036]/0.05. The percentage of drug release was calculated by using the Equation (1), in which Mt is the amount of drug released at any time (t) and M0 is the total mass of drug loaded into the hydrogel.

$$\% \text{ Released} = \frac{Mt}{M0} \times 100\% \quad \text{Equation (1)}$$

To maintain constant the volume and mass of the delivery systems, after measurements, the aliquots were returned to their respective solutions.

2.5 Characterization

Rheological analysis: Dynamic shear oscillation measurements were used to characterize the viscoelastic properties of the hydrogels. The rheological measurements were carried out with a Rheometer Anton Paar Physica MCR-101 using parallel plates of 25 mm of diameter and plate-to-plate distance of 6 mm. The temperature dependence of the storage (elastic) modulus was determined by oscillatory shear deformation (dynamic rheological observations) with temperature scan ranging from 60 to 25 °C (cooling rate 1 °C min^{-1}) at constant frequency of 10 Hz and constant shear strain ($\gamma = 0.5$).

Swelling and hydrogel stability: The hydrogels were cut, weighted and dried (45 °C). The samples were placed in beakers filled with 2 mL of aqueous solutions buffered at pH 5 (acetic acid/sodium acetate), pH 7 (potassium phosphates) and pH 9 (sodium bicarbonate/carbonate), at two different temperatures (25 and 37 °C). The materials were weighted at different intervals of time to measure the percentage of absorbed solution. The swelling test finished when the materials maintained the same weight after 3 consecutive measurements.

The in vitro degradation behavior and the stability of the PEGD:CA/GEL/TR polymeric drug carrier was investigated for a period of 2 weeks. Experiments were conducted in three different buffers (pH = 5, 7 and 9) at two different temperatures (25 and 37 °C). Pictures at different intervals of time from the same focal length for its comparison were taken.

The swelling process was monitored using the following equation:

$$S\ (\%) = \frac{(Ws - Wd)}{Wd} \times 100\% \quad \text{Equation (2)}$$

Where: Ws is the weight of the swollen hydrogel at different intervals of time and Wd is the weight of dry sample.

Kinetic analysis: The Rigter & Peppa's equation [31] (Equation (3)) was used to obtain the kinetic parameters of tramadol released. The constants n and k which are characteristics of the mechanism of transport and the nature of the material, were obtained using the Equation 3 after linearized [32-33]. The experiments were done in triplicate and the standard deviations were calculated.

$$\frac{M_i}{M_\infty} = kt^n \qquad \text{Equation (3)}$$

Morphological analysis: In order to see the pores of the crosslinked network the PEGD:CA/GEL and PEGD:GTA/GEL hydrogels were swollen with deionized water. Their morphology was examined by using a Scanning Electron Microscopy (SEM, FEI Quanta200) at 400, 800, 1600 and 3000 X magnifications.

Thermal analysis: In order to determine the thermal stability of the hydrogels, thermo gravimetric analysis (TGA) were carried out by using a TA Instrument (Q500). During analysis, the nitrogen and air fluxes were maintained at 20 and 40 ml/min, respectively. The samples were placed in an aluminum pan and heated from room temperature to 500 °C with a heating rate of 10 °C/min and were analyzed in two forms: a) after swelling to their maximum capacity with deionized water at 25 °C and b) after drying at 45 °C for 48 h.

2.6 Statistical Analysis

All of the data represent the mean values ± standard deviation of the independent measurements. Statistically significant differences between pairs of mean values were determined with ANOVA. Experiment design was done in software Minitab 16; the response parameter was the percentage of tramadol released. We used a factorial design 3^2, with the following factors: percentage of PEGD (5-15 %), CA concentration (17.6-35.2 mM) and reaction temperature (40-70 °C). Mean differences with p-values ≤ 0.05 were considered statistically significant, the p value of CA was 0.03 and for PEGD was 0.05. These results indicated that both parameters have important effect during the release process. The temperature did not show an important effect, so it was kept constant throughout the experiments (60 ºC).

3. Results and Discussion

3.1 Mechanism

The proposed mechanisms for the crosslinking of PEGD:CA and PEGD:GTA hydrogels are shown in Fig. 1. The Fig. 1a shows the crosslinking mechanism among the PEGD and CA. It is suggested that the physical interactions between the positively charged terminal amine groups (NH_3^+) of PEGD which were protonated by the acidity of the solution (pH 5), and the deprotonated and negatively charged carboxylic groups (COO^-) of CA govern the process. The weak nature of these physical interactions produce a polymeric material with low mechanical strength. But, by the other hand, the high flexibility of this 3D network, might favor the hydrogel swellability, mainly due to the lower stiffness on the bonds which are formed primarily by intermolecular forces, such as attractive interactions (NH_3^+ and COO^-) and hydrogen bonds among the non-ionized carboxylic acid functional groups of GEL and CA [34]. Fig. 1b shows the chemical crosslinking mechanism between the terminal amine groups of PEGD and the aldehyde groups of GTA, in this case the mechanism of bond formation is carried out by condensation of the primary amine of PEGD which in acid medium produces an imine (Schiff base) [35–37]. The formation of the covalent bonds promotes the increasing of the mechanical strength of the polymeric material, which consequently has an impact on its swelling properties, mainly due to the formation of a less flexible network. However, it is important to point out that for both hydrogels the functional groups of the polymer chains have also an important contribution in the hydrogel swelling as it will be shown in the next sections.

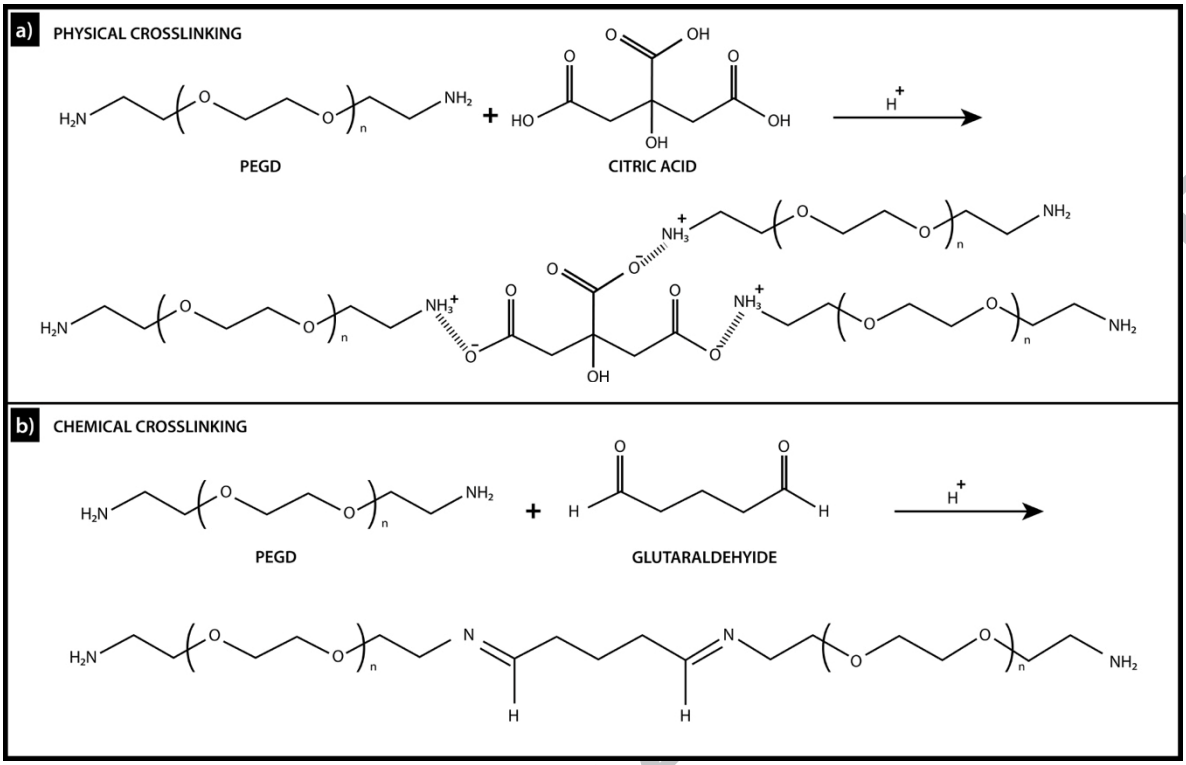


Figure 1

3.2 Rheological Analysis

The physical structuring of the hydrogels depends on the temperature, the cooling rate and the gelation time. Therefore, after the reaction of synthesis, the hydrogels were controllably cooled to room temperature. This experiment provided information on the viscoelastic properties of the materials by measuring the mechanical response of the samples when they were deformed under a periodic strain. Fig. 2 shows the elastic modulus G' (also real or storage) and the viscous modulus G'' (also imaginary or loss) both obtained during the dynamic and static isothermal oscillatory tests. The measurement were carried out within

the linear viscoelastic region of the materials, ensuring that the measured hydrogel properties are independent of the magnitude of the imposed strain or stress [38].

3.2.1. Dynamic Cooling Analysis

The values of G' and G'' for PEGD:CA/GEL and PEGD:GTA/GEL hydrogels at crosslinking ratios of (1:1) and (1:2), and those corresponding to the TR composites at the crosslinking ratio of (1:2) are shown in Fig. 2a and 2c respectively. It is observed that the physically crosslinked hydrogel (Fig. 2a) show higher values of the elastic modulus than the chemically crosslinked gel (Fig. 2c). To explain this behavior it is important to mention that the main component (around 90 % in weight) of PEGD:CA and PEGD:GTA hydrogels is gelatin, which acts as hydrogel vehicle. It is well known that gelation of gelatin is produced when is cooled below its melting point (30-35 ºC) forming a physically crosslinked network [39]. Therefore the increasing of $G´$ during the cooling process is attributed to the physical structuring of the gels. The $G´$ increases with decreasing temperature until reach the maximum value at 25 ºC (1.75 MPa for PEGD:CA/GEL and 0.75 MPa for PEGD:GTA/GEL) indicating that the CA crosslinked polymer forms a stronger hydrogel. This might be due to the high density of CA functional groups (three carboxylic groups/molecule) that primarily can interact with the amine groups of PEGD, but also with the solvent molecules (water) and/or the amine groups of gelatin, forming bonds of physical nature (see Fig. 3).

When TR was added to both polymeric matrices the strength of PEGD:GTA/GEL hydrogels becomes quite similar to that obtained for PEGD:CA/GEL (CA-$G´$=1.3 and GTA-$G´$=1.2 MPa, respectively). This result denotes the substantial contribution of TR to

PEGD:GTA/GEL structuring, while for CA:GTA/GEL seems to have no major effect. These results indicate that the hydrogels structuring depends on the temperature but also on the chemical nature of the polymeric matrix. It is also interesting to observe that both hydrogels at all the studied temperatures show higher values of G´ when compared with G″, indicating that the contribution of the elastic segments in both materials is more significant than in the viscous portion. This could be an advantage, because a more flexible matrix might provide better swelling or release properties. It is worth mentioning that the final structure of the gels determines their mechanical properties, which in turn will have an impact on the gel swelling, gel stability and on the drug release kinetics, as it will be shown in the next sections.

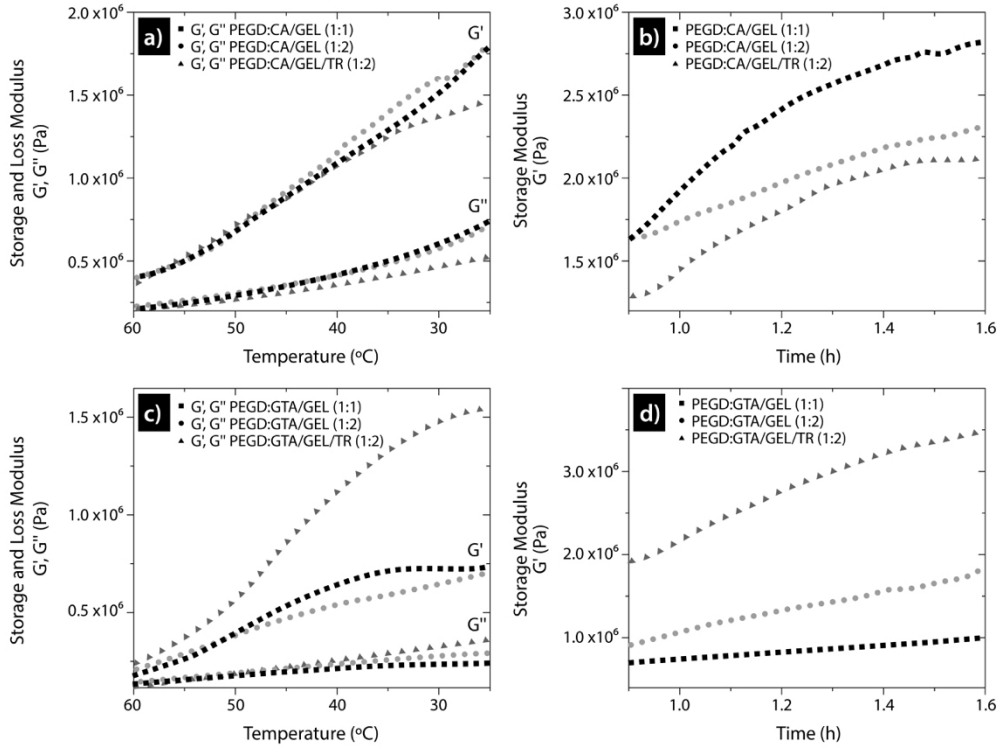


Figure 2

3.2.2. Isothermal Analysis

The isothermal oscillatory test at 25 ºC for PEGD:CA/GEL and PEGD:GTA/GEL is shown in Fig. 2b and 2d respectively, on this studies only G' is given for an easier comprehension of the data. On the graphs, it can be seen two effects: 1) the gel structuring continues over the time, and 2) for PEGD:CA/GEL the addition of TR and crosslinker at (1:2) stoichiometric ratio promoted the decreasing of G', while for the PEGD:GTA/GEL hydrogel increased it. These last results can be explained in terms of a combined effect induced for the physical interactions (those produced during gel structuring) and the formation of covalent bonds between the amine groups of gelatin and the aldehyde groups of GTA (in excess in this hydrogel), which seems to be favored at room temperature. To prove our hypothesis, hydrogels crosslinked with GTA at (1:5) ratio were synthetized (results not shown). After cooling them at 25 °C, it was obtained an extremely stiff hydrogel. The hardness of the polymeric materials at (1:5) PEGD:GTA ratio was so high that was not easy to separate them from the vessel of reaction. Based on this result, and although the (1:5) chemically crosslinked hydrogels were the materials with higher stiffness, were not selected for this work, because their rigidity can interfere with the drug release profiles [41], which is not desirable for drug controlled release applications. On this regard, the (1:2) PEGD/crosslinking ratio hydrogels provided more adequate properties for this purpose.

It was also observed that although the chemically crosslinked hydrogel showed an increase in the storage modulus, their values were always lower than those obtained for the PEGD:CA/GEL hydrogels. Highlighting the importance of the gel physical structuring which in our opinion contributes far more to their viscoelastic properties that the chemical crosslinking alone.

Unexpected was to find that the G´ value of PEGD:CA dropped to 2.1 MPa after the addition of TR while for the PEGD:GTA increased up to reach a maximum value of 3.5 MPa. Thus, the encapsulation of TR into the pores of PEGD:GTA heterogeneous network (Fig.3b-III) seems to provide the hydrogel certain capability to absorb energy.

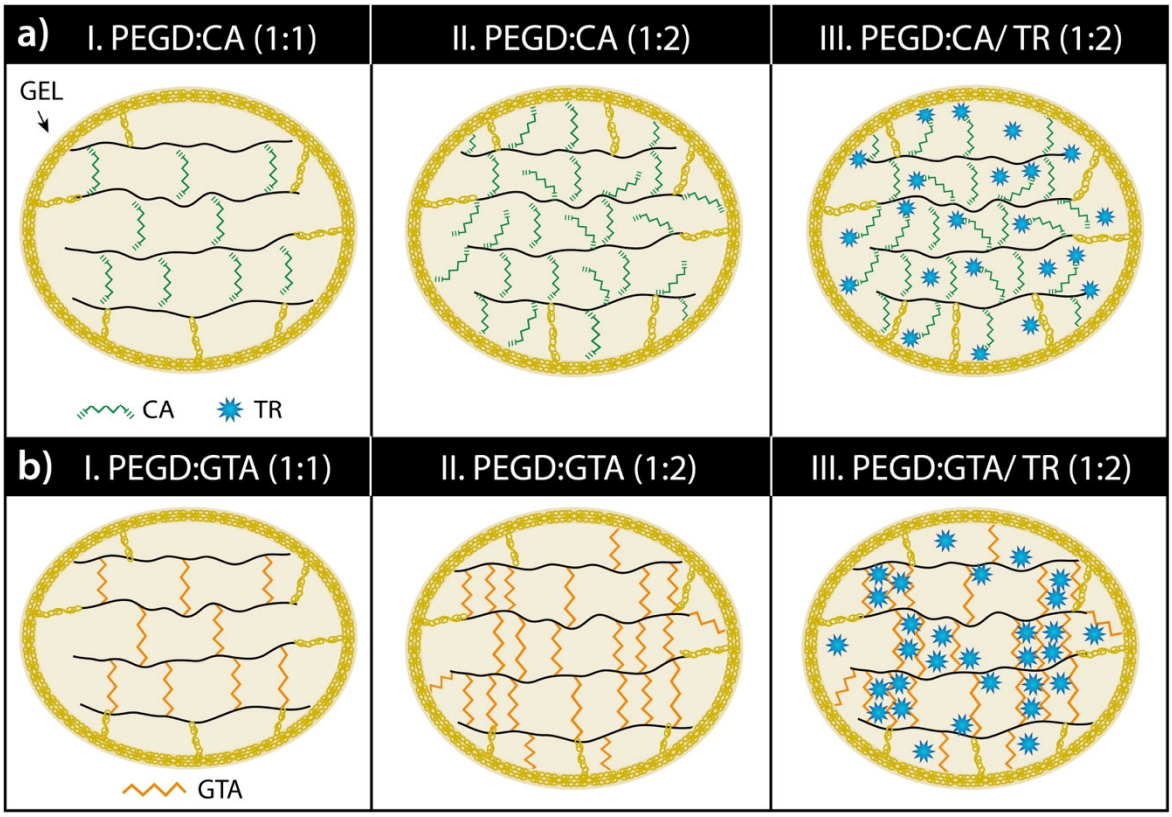


Figure 3

3.3 Thermal Analysis

The thermal gravimetric analysis was performed to investigate the stability of the hydrogels in aqueous media. The results of differential thermal analysis (DTA) of PEGD:CA/GEL and PEGD:GTA/GEL at different molar ratio of crosslinkers (CA or GTA) are presented in Fig. 4. The thermal behavior of PEGD:CA/GEL hydrogels is shown in Fig. 4a. It is

observed that the maximum loss of water occurs at 65 °C for sample with crosslinking ratio of (1:1) while for sample with crosslinking ratio of (1:2) was found at 104 °C. These results indicate that the excess of CA has a significant effect on the water desorption's temperature, as we explained before, may be due to the increased interactions of free carboxylic acid functional groups (-COOH, $-COO^-$) with GEL and water that rise the water retention capacity. Also it is interesting to notice that the water desorption is achieved in one step (just one peak is seen) confirming the homogeneous nature of the PEGD:CA/GEL porous structure (Fig. 3a). On the other hand, for the (1:1) PEGD:GTA/GEL hydrogels the temperature of the maximum loss of water was observed at 91 °C (26 °C higher that CA hydrogels). This ratifies the formation of a more crosslinked and heterogeneous network, which promotes a stronger retention of water molecules in its different domains (Fig. 4a and 4b). This effect was confirmed with the hydrogel crosslinked at (1:2) GTA ratio, where several important losses of water around 89, and 112 °C were seen (Fig. 4b). The highest temperature corresponds to the more crosslinked network. All this results confirm that PEGD:GTA/GEL hydrogels containing an excess of GTA, produced a heterogeneous structure with the capability of trap water in different layers of the 3D network. Hence, the water molecules trapped in their surface porous is desorbed at less higher temperatures compared with those adsorbed or trapped in the internal or smaller pores of the polymeric network (Fig. 4b).

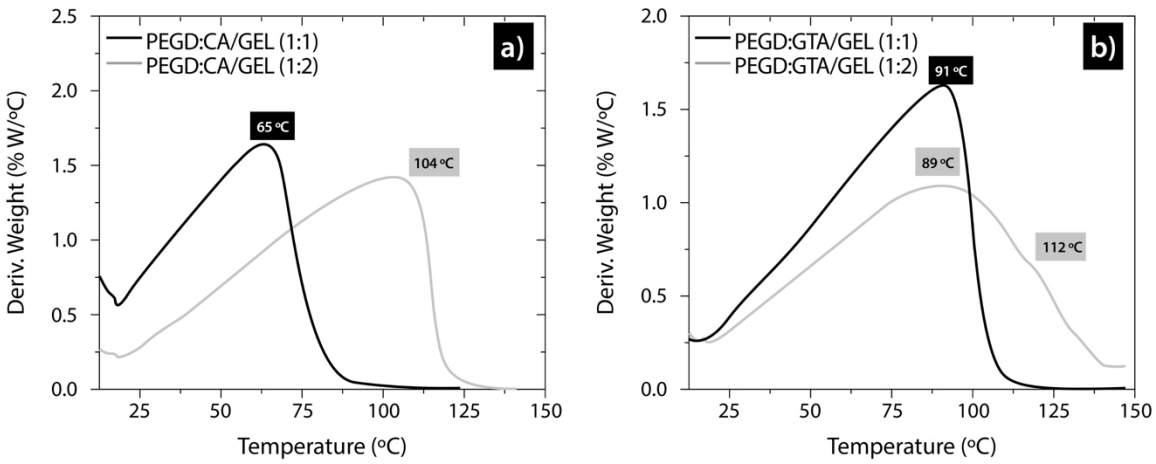


Figure 4

In addition, TGA analysis of the hydrogels in dry form were obtained to identify their degradation temperatures and stability. In order to have a suitable reference to be compared, the thermograms of the pure compounds were obtained. The degradation temperatures were found at: 330 and 393 °C for PEGD, 274 and 316 °C for GEL, 212 °C for CA and 253 ºC for TR (Fig. 5a). In a first step the thermograms of PEG:GTA/GEL and PEG:CA/GEL hydrogels were obtained (results not shown). However, the main signature that was found in those results corresponded to GEL, which is the main component of the polymeric matrices. No changes in the degradation temperature were observed when comparing the thermograms of composites with that of pure GEL, confirming the physical nature of the interactions formed during the hydrogels structuring. Therefore, to have information about the thermal stability of PEGD:GTA and PEGD:CA polymers and to avoid the screening of their interactions, we made the synthesis of these hydrogels but without using the support matrix (GEL), at crosslinker ratios of (1:1) and (1:2) respectively, this last with and without TR. The results are shown in Fig. 5b and 5c. It can be seen that

the crosslinked polymers decreased the degradation temperature by approximatively 8 °C (CA:PEGD 385 ºC ±3 °C, GTA:PEGD 384 °C±1 °C) with respect to that observed for pure PEGD (393 °C), thus, by comparing with the latter, both hydrogels showed less thermal stability. In addition, at (1:2) crosslinking ratio, the shoulder at 335 °C was not observed. The absence of this degradation peak is attributed to the formation of hydrogels with higher crosslinking density.

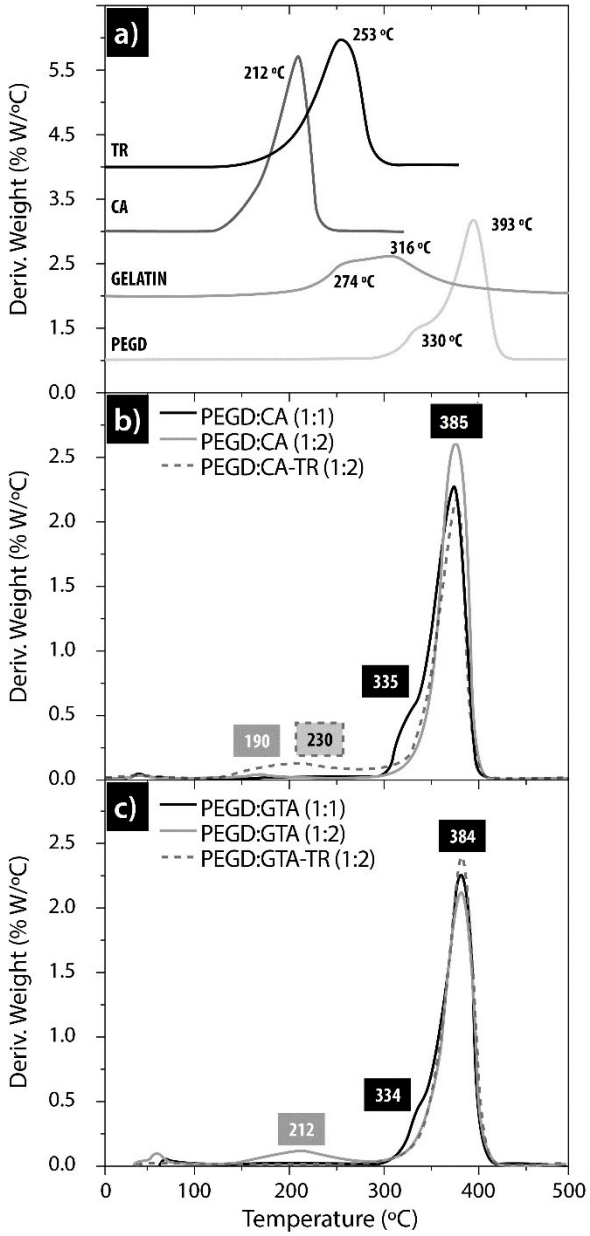


Figure 5

3.4 Swelling and Hydrogel Stability

The swelling behavior of hydrogels is important in biomedical and pharmaceutical applications. The precise control of these properties is particularly important for polymer drug delivery systems and during the production of hydrogel super-absorbents. On this

regard, it is known that the swelling capacity of non-ionic hydrogels depends on the chemical composition of the polymers and often they do not respond to external pH changes, however, when ionic moieties are incorporated into the hydrogels, the swelling depends on the chemical composition but also on the pH of the surrounding medium. Additional factors that might influence the responsiveness behavior of hydrogels are the density of crosslinking, type of formulations (functional groups), molecular weight and temperature [39], [41]. Thus, the pH-temperature-responsiveness of PEGD:CA/GEL and PEGD:GTA/GEL hydrogels at (1:2) PEGD/crosslinker ratio was evaluated, the results are shown in Fig. 6a-c. It is noticed that at 25 ºC and pH 5 and 9, both hydrogels exhibited the same slight differences in the swelling percentage (~500-600 %). However, when the hydrogels were swollen at pH 7 the maximum swelling capacity of PEGD:CA/GEL increased by 30 %, giving rise a swelling maximum of 650 % (similar to PEGD:GTA/GEL). The fact that both hydrogels show the same swelling capacity at acid and basic media indicates that the swelling governing mechanism is mostly dependent on the polymers chemical composition and the density of crosslinking (there is not influence of the functional groups). Even the addition of ionic moieties in the PEGD:CA/GEL network not influence the response of the hydrogel. In fact, at room temperature, an excess of ionic species (H^+ or ^-OH) seems limit the diffusion of water into the polymeric matrices, while the balance of them slightly favor it.

On the other hand, when the test was carried out at 37 ºC, important changes in the swelling capacity was observed for both hydrogels. The highest swelling percentage was obtained at pH 5 for the PEGD:CA/GEL hydrogel (2000 %), but interestingly at pH 7 both hydrogels maintained the same swelling behavior which is almost the double of those observed at 25

ºC (~1300%,). At pH 9 slight differences in the water absorption capacity were also seen. It is important to notice that under acidic and basic conditions the higher adsorption capacity of PEGD:CA/GEL hydrogel prevailed which confirm the elastic nature of the polymer. The impact of temperature in the hydrogels swelling capacity can be attributed to the increasing of hydrogels chains flexibility, which in turn relaxes the polymeric matrices allowing the diffusion of higher amount of water inside of the 3D networks. This behavior is classic of thermo-responsive hydrogels. However as was previously described, PEGD:CA/GEL and PEGD:GTA/GEL hydrogels are formed by a PEGD crosslinked network and a gelatin polymeric entanglement. Therefore, the swelling behavior observed at 37 ºC for the PEGD:CA/GEL hydrogel point out to a combined contribution given for the movement of the polymeric chains but also for the possible interactions of the protonated and un-protonated functional groups of gelatin and citric acid with the ionized groups of water.

The maximum swelling of PEGD:CA/GEL hydrogels observed at 37 ºC was obtained as follows: pH 5 > 9 > 7 with percentages of 2063 %, 1500 % and 1270 %, respectively. This behavior can be explained in terms of the ionizable structure of gelatin, which has a PKb of ~ 6.5 and pKa of ~4.7 for the NH_3^+ and COOH ionizable groups [42]. Hence, the predominant form of the gelatin depends on the pH of the solution. In strong acidic media (pH<3), the positive (NH_3^+) and the non-dissociated (COOH) groups of gelatin prevail; while in strong basic media (pH>8) the NH_2 and COO^- are the main species; when the pH of the media are between the pH of PKb and PKa (4.0<pH<7.0) the species are any of those mentioned above. On this basis, the higher swelling of the PEGD:CA/GEL hydrogels at pH 5 (Fig. 6a) can be attributed to the flexibility of the polymeric chains that allow the diffusion of water molecules. Once the water get inside of the porous matrix, the positive

(H^+) and negative (^-OH) species of dissociated water, as well as those of the acetate buffer (CH_3COOH/CH_3COO^-) can induce attractive forces between the ionizable groups of gelatin, PEGD and/or citric acid (NH_3^+, COO^-). The overall effect is the formation of a flexible network, and an increase on the swelling capacity of the hydrogel. At neutral pH (Fig. 6b) both hydrogels exhibited the same swelling behavior showing not significant contribution of the ionizable groups. At pH 9 (Fig. 6c), the predominating ionic specie in the hydrogels are the carboxylate groups COO^-, which probably interact with the dissociated species of water, in this case the interactions of the polymeric network with the dissociated species of bicarbonate/carbonate buffer ($HCOO_3^-/CO2^{-2}$) is limited because they show similar charges. However, it is important to remark that these interactions are complex by nature, if other groups of the polymer chain may also be exposed and interact with the ionic moieties.

For PEGD:GTA/GEL hydrogels was observed almost the same swelling response at the three pH´s, indicating that this polymeric network is not easily affected by changes in the pH of the swelling medium, most probably because the high crosslinking density of the hydrogel and the heterogeneity of its 3D microstructure, that limit the diffusion of water and the expansion and the mobility of the polymer chains.

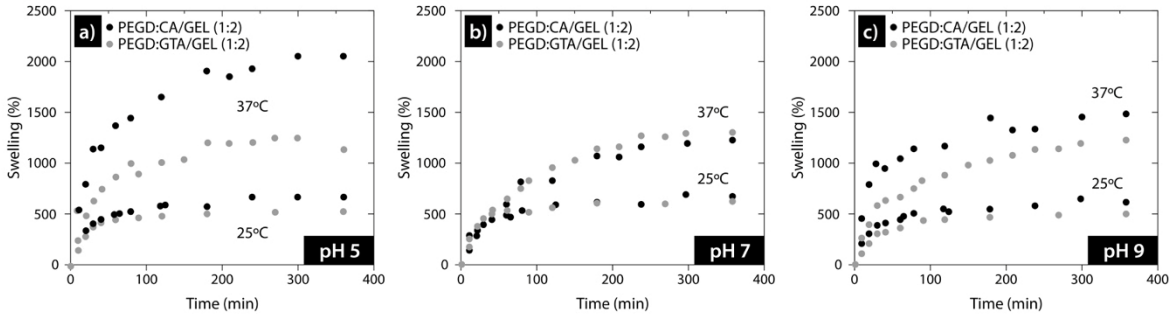


Figure 6

Once conducted the swelling test, the degradation process of the hydrogels was evaluated by immersing the polymeric materials in phosphate buffered solutions at pH 7. Pictures at different intervals of time and two temperatures (25 and 37ºC) were taken for PEGD:CA/GEL (Fig. 7a-I and 7a-II) and PEGD:GTA/GEL (Fig. 7b-I and 7b-II). From these figures, changes in mass loss can be visually confirmed for both hydrogels. It is seen that at 25 ºC, PEGD:GTA/GEL maintains its shape up to 30 hours, while the PEGD:CA/GEL hydrogel under the same conditions loss a significant amount of mass. For this last material, the polymeric matrix started to break up at ~20 h and after 30 h, part of the material is solubilized due to the hydrolysis processes that cause the breaking of intermolecular bounds inside the polymeric matrix, accelerating its degradation. The higher stability of PEGD:GTA/GEL can be attributed to the formation of the chemically crosslinked network (This result agrees well with the rheological analysis explained above).

The temperature has an important effect on the stability of hydrogels as in shown in Fig. 7b. At 37 °C, both materials begin to lose their structure at ~ 8 h. This behavior is produced for the high relaxation of the polymeric chains that allows the entrance of water molecules into the 3D polymeric networks, facilitating its hydrolytic degradation. Not significant differences on the swelling behavior was observed in the polymeric matrices, which is attributed to the pH of the degradation solutions (neutral). These results agree well with those obtained during the swelling test (Fig. 6b). Table 2 reports the final time at which the complete solubilization of the materials was observed. It was found that when the temperature increased at the body temperate (37 °C), the degradation time of the materials is reduced by approximately 80 % with respect at that observed at room temperature (25 ºC).

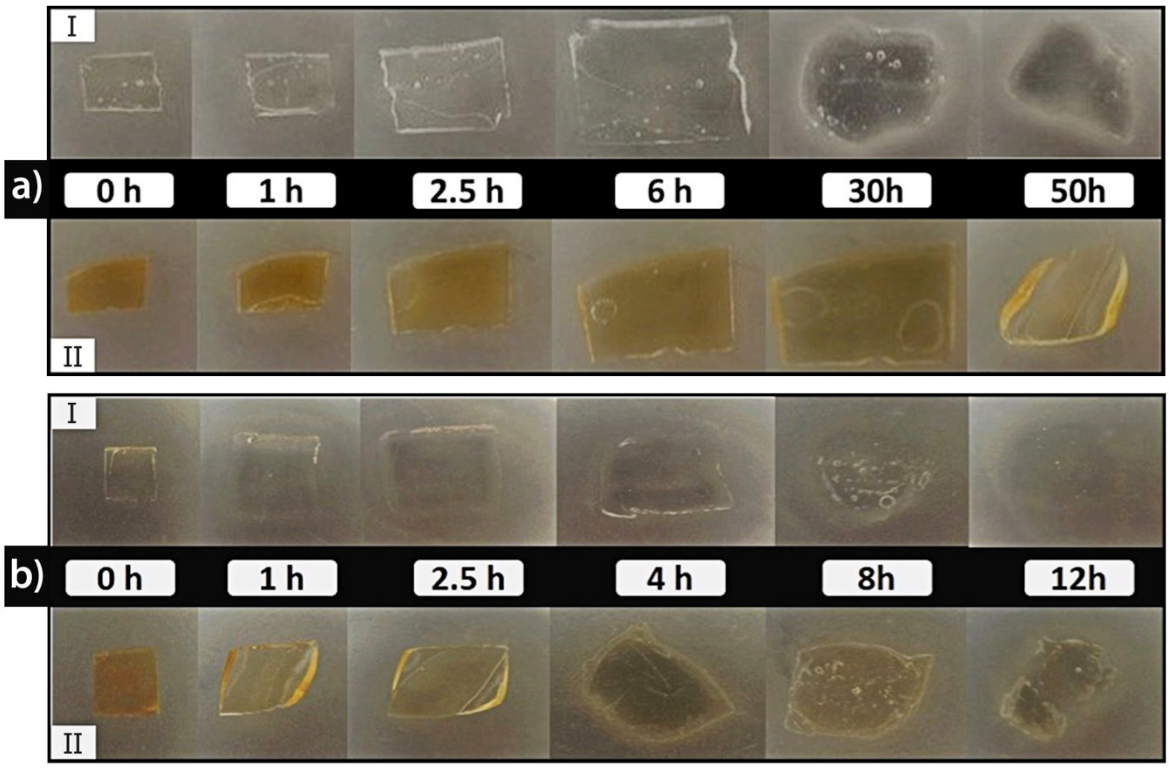


Figure 7

Table 2. Degradation time analysis of PEGD:CA/GEL and PEGD:GTA/GEL hydrogels in phosphate buffer pH 7 at 25 and 37 °C (average values).

PEGD:CA/GEL		**PEGD:GTA/GEL**	
25 °C	**37 °C**	**25 °C**	**37 °C**
90 h	15 h	180 h	20 h

3.5 In Vitro Drug Release Study, Morphological Analysis and Kinetics

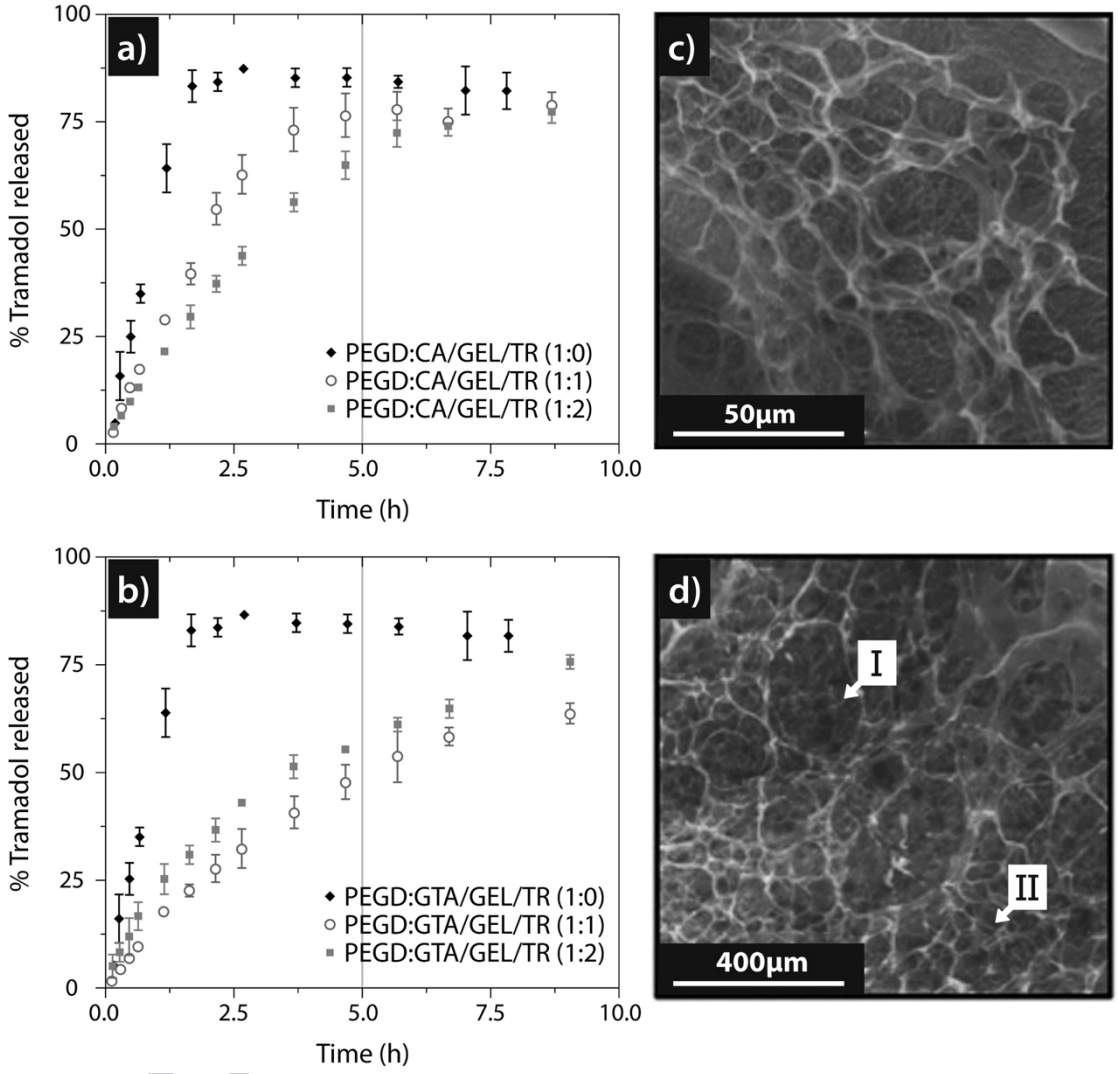


Figure 8

Fig. 8a and 8b show the release profiles of TR composites at 37 ºC as function of the PEGD:crosslinker ratio. The hydrogel labeled as (1:0) does not contain crosslinker, and acts as reference to determine the effect of the polymeric network formed for the entanglements and physical interactions of GEL-GEL and PEGD-GEL. The kinetic curves of these materials show a low level of drug dosage. At ~ 2 h the 87.5 % of TR is released from these physically structured hydrogels, showing the poor capability of (1:0) PEGD/GEL/TR hydrogel to control the release of the drug. The formation of a crosslinked hydrogel by

adding the crosslinking molecules (GTA or CA) causes a dramatic change in the release profile. However it is important to mention that during the first hour the polymeric materials behave similarly, releasing approximately 12 % of the loaded TR through the "burst" mechanism [43]. After this time, the release profiles show important changes attributed to each polymer formulation type.

The TR release behavior of physically crosslinked PEGD:CA/GEL hydrogels at (1:1) and (1:2) PEGD:CA ratios are shown in Fig. 8a. The capability of TR dosage at 5 h was compared. It was noticed that (1:1) PEGD:CA/GEL released ~76 % of TR which represents 11 % less than the reference material (without crosslinker); while the (1:2) PEGD:CA/GEL released ~65 %. These results confirm the formation of a crosslinked hydrogel and the important role that CA plays in the formation of the 3D network. The fact that the less crosslinked network gives a quicker drug release than the once of the higher crosslinked network, indicates the high ability of PEGD:CA/GEL to induce the polymer chain-relaxation. It is also important to mention that the swollen capability of the hydrogel has certain influence too, because the relative mobility of the solvent and the drug increases in the presence of a macromolecular relaxation.

On the other hand, the release behavior of the hydrogels chemically crosslinked (PEGD:GTA/GEL) are shown in Fig. 8b. In this case, the percentage of TR released for the (1:1) PEGD:GTA/GEL was ~48 % (which is 40 % less than the reference material), while for the (1:2) PEGD:GTA/GEL was ~51%. The differences are not significant (contrary to that observed for CA hydrogels). This is attributed to the formation of covalent bonds which produced a more rigid and chemically crosslinked mesh, the lower flexibility of the chain reduced the relaxation capability of the hydrogel, giving rise to a lower kinetics of TR

release. In these materials approximately 40 % of TR remains trapped in the hydrogel after 10 h.

As expected, the results confirm that the chemically crosslinked network induces a better control during the release processes, however it is important to highlight that the network physically crosslinked with CA also show high ability to produce the drug dosage, with the additional advantage of being biocompatible, less toxic and non-expensive. At this point it is important to remind that GTA has proved to be a compound of high toxicity that even at low concentration shows cell-growth inhibition [44].

In addition, the release kinetic's rate of TR in the physically crosslinked CA hydrogel can be improved by increasing the amount of CA in the polymeric matrix. This was confirmed by comparing the differences in the percentages of released TR in both networks at 5 h. It was found that at (1:1) PEDG:crosslinker ratio the amount of released TR differs 28 % (comparing both hydrogels), while for the (1:2) ratio differs 14 %. Finally, it is important to remark that in CA hydrogels the physical interactions play an important role as was explained during the rheological analysis (Fig. 3a). These interactions seem improve the dosage of the drug, retaining it in the polymeric mesh for longer periods of time, this behavior is similar to that observed in the thermal analysis in which the addition of CA at (1:2) PEGD:CA ratio produced a red shift of 39 ºC during the water evaporation test (Fig. 4a).

Fig. 8c and 8d show the morphological analysis performed to both polymeric networks. To assure the maximum expansion of the 3D mesh both materials were swollen at their maximum capacity. However, during the experiments, the hydrogels gradually lost certain amount of water, mainly due to the electrons beam which heated the sample, consequently,

when taking the images, the hydrogels had different levels of swelling. Fig. 8c corresponds to a SEM micrographs of (1:2) PEGD:CA/GEL, which show a regular porous network with pores size in the order of microns (~ 20 µm). Fig. 8d shows the micrograph of (1:2) PEGD:GTA/GEL, the porous mesh looks highly porous, a close inspection of this image show that this materials contains at least two different domains (arrowed), which comprise zones with porous around 150 µm and pores in the order 30 to 5 µm, which confirm the hypothesis of Fig. 3.

Fig. 9 describes the suggested mechanism of TR release. The drug release begins when the hydrogel is in contact with an aqueous medium, after which, the swelling process of hydrogel starts. At this initial time the drug that was adsorbed onto the hydrogel surface is quickly released following the burst mechanism. After hydrogel swelling the drug diffusion trough the pores of the polymeric matrix domain the release process. When the hydrogel reaches the maximum swelling, the drug release is driven by both passive diffusion and degradation phenomena. It is clear that after maximum swelling, the volume expansion that suffer the hydrogels increase the mobility of their chains and at approximately 12 h for PEGD:CA/GEL and 20 h for PEGD:GTA/GEL the networks start to break (all this at 37 ºC), the fragmentation of the hydrogels continues in time until reach their complete solubilization.

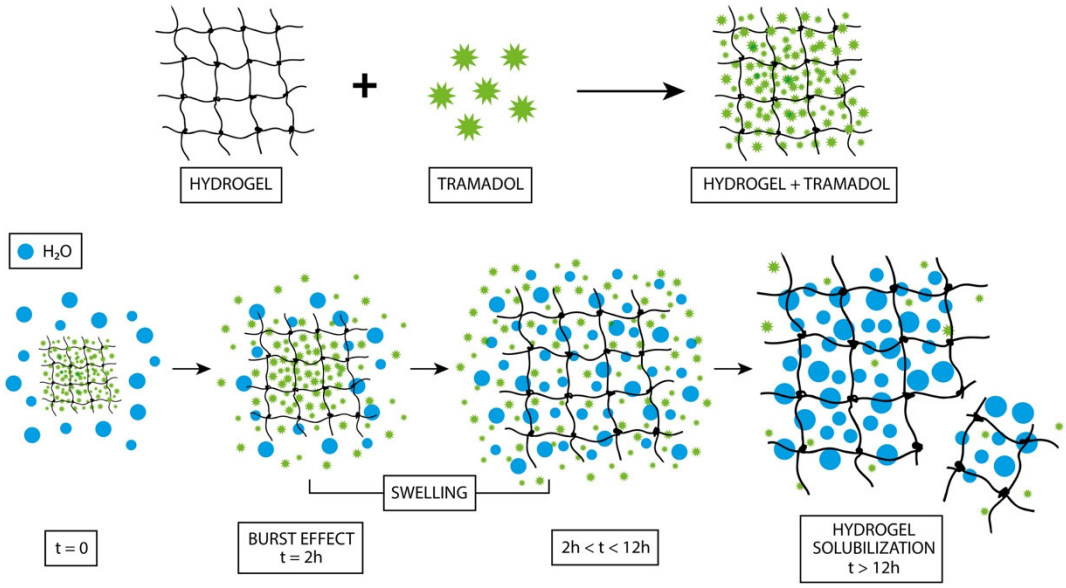


Figure 9

The kinetic parameters of release were obtained by using the Ritger & Peppa's equation (Equation (3)). In the equation, the values of "n" determine the release mechanism and "k" the material nature. Therefore, after linearizing the equation, the kinetics values of the different materials were modeled and the results are shown in Table 3. The characteristic constant of the material "k" has very approximate values of ~0.7 which indicates that the materials have similar properties despite having both concentrations of CA. This does not occur with PEGD:GTA/GEL where the constant "k" is different for each material with values from 0.70 ± 0.002 to 0.95 ± 0.005, this is because they might have differences in their microstructure. The arrangement of the materials depends entirely on the crosslinking, it was observed a significant change in the analysis of swelling and stability when crosslinking is chemically produced. The morphological structure of both hydrogels is quite similar and can be seen in the micrographs of Fig. 8c and 8d, wherein the materials show a reticulated porous internal structure.

The "n" values for PEGD:CA/GEL remains close to 0.9, indicating an anomalous mechanism, which is characterized by both contributions: drug diffusion and polymer chain relaxation. For materials synthesized with GTA the constant "n" varies in both hydrogels, for PEGD:GTA/GEL (1:1) the value is n=0.96 ± 0.281, indicating a "zero order" mechanism, on which the release process is regulated by the water entering into the porous network. On the other hand in PEGD:GTA/GEL(1:2), it was found a "n" value of 0.74 ± 0.023, which indicates an anomalous mechanism. The two different release mechanism can be attributed to the highly heterogeneous structure of GTA hydrogels.

The anomalous mechanism is predominant in both (1:2) PEGD:CA or GTA hydrogels, which coincides well with the findings reported in the literature [24-32], in those is stated that the anomalous mechanism is predominant in TR loaded hydrogels. Some of them are the reports of Acosta et al. [26] and Anirudhan et al. [27] which synthesized alginate/chitosan matrices (n=0.53 to 0.84) and PEG/PVA polymeric micelles (n=0.89) respectively.

Table 3. Kinetic parameters of the hydrogels with Citric Acid and Glutaraldehyde.

Kinetic parameters	PEGD:CA (1:1)	PEGD:CA (1:2)	PEGD:GTA (1:1)	PEGD:GTA (1:2)
n	0.94±0.08	0.86±0.051	0.96±0.281	0.74±0.023
k	0.63±0.005	0.73±0.004	0.95±0.005	0.70±0.002
R^2	0.98±0.018	0.99±0.006	0.96±0.018	0.98±0.056

4. Conclusions

The benefits of use biodegradable and biocompatible polymers for the controlled release of therapeutics is currently well stablished. In the literature there are a large number of reports that claim the benefits of new formulations, however only a small number of them have

been implemented in the pharmaceutic industry. The reasons are often related with the use of high cost precursors and complicated methods of synthesis. In this work we propose an alternative by fabricating a new temperature-responsiveness hydrogel, our synthesis method is easy to implement and uses low cost precursors. PEGD was physically crosslinked with citric acid to obtain a 3D network, which was then encapsulated in a gelatin matrix that provides a viscoelastic consistency to the template that facilitates its handling. The capability of PEGD:CA/GEL hydrogel to serve as controlled release system was evaluated by encapsulating TR into the 3D porous network. The results of our research showed that if the synthesis parameters of PEGD:CA/GEL hydrogel are handled, the gel properties can be changed in terms of the degradation times, mechanical properties and release kinetics. The performance of TR loaded PEGD:CA/GEL hydrogel was compared with a hydrogel network chemically crosslinked with GTA. Both hydrogel matrices were thermally and physicochemically characterized by using thermal, rheological, morphological and spectroscopic methods. In addition swelling, stability and kinetics studies were conducted to investigate the effect of crosslinking ratio, temperature and pH in the polymeric networks. Through these studies, we showed that the physically crosslinked hydrogels are more flexible than the chemically crosslinked ones and surprisingly the physical forces were strong enough to maintain the drug dosage in similar way that the chemically crosslinked network. It was demonstrated that PEGD:CA/GEL and PEGD:GTA/GEL swelling is temperature-dependent. The pH of the medium showed influence only in the PEGD:CA/GEL hydrogels, being more important at the body temperature (37 ºC) and acid media (pH 5), which for biological applications gives an advantage of a dual response. Thermal analysis revealed that the addition of TR increases the stability and the mechanical properties of hydrogels. During drug release kinetics studies it was found that the drug

release mechanism for PEGD:CA/GEL follows an anomalous mechanism characterized by two contributions: drug diffusion and polymer chain relaxation while for the PEGD:GTA/GEL hydrogels is dependent of the crosslinker density, showing for (1:1) and (1:2) PEGD:GTA ratios a "zero order" and anomalous mechanisms, respectively. Although the present research is targeted at controlled drug release, the PEGD:CA/GEL hydrogels are potentially suited for a wide range of in vivo biomedical applications, mainly due to the high biocompatibility and biodegradability of PEGD and gelatin with the additional advantage that the addition of CA might provide them cytocompatibility.

Acknowledgements

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Figure Captions

Scheme 1. Tramadol molecule: a) non-ionized form (pH < 9) and b) ionized form (pH < 9). Data obtained from the speciation diagram at [TR]= 500 ppm and PKa=9.41 [29-30].

Fig. 1. a) Mechanism of physic crosslinking with Citric Acid. b). Mechanism of chemical crosslinking with Glutaraldehyde.

Fig. 2. Rheological behavior during the cooling process. Dynamic oscillatory test with cooling ramp from 60 to 25 °C and rate of 1 °C/min, a) PEGD:CA/GEL (1:2) and c) PEGD:GTA/GEL (1:2). Static isothermal oscillatory test (25°C), b) PEGD:CA/GEL (1:2) and d) PEGD:GTA/GEL (1:2).

Fig. 3. Proposed structure of hydrogels synthetized with different polymer/crosslinker ratio, before and after the addition of TR. a-I) (1:1) PEGD:CA suggest the formation of a polymeric network with large pores and homogeneous domains. a-II) (1:2) PEGD:CA shows the unreacted CA molecules, which are probably interacting with gelatin. a-III) PEGD:CA/TR, free CA molecules interacting with amine groups of gelatin and/or tramadol. b-I) (1:1) PEGD:GTA shows a polymeric network with large and small pores (formation of heterogeneous domains). b-II) (1:2) PEGD:GTA shows a highly crosslinked network, when GTA percentage increases, they have the propensity to involve intra cross linkage within gelatin as availability of amine groups of PEGD is very less compared with gelatin. b-III) (1:2) PEGD:GTA/TR shows the possible entrapment of TR molecule inside of the highly crosslinked network.

Fig. 4. Swollen hydrogels behavior during evaporation of water: a) PEGD:CA/GEL and b) PEGD:GTA/GEL at different concentrations of crosslinking agent.

Fig. 5. Thermogravimetric analysis of a) Pure compounds, b) PEGD:CA (1:1), (1:2) and (1:2) with TR and c) PEGD:GTA (1:1), (1:2) and (1:2) with TR.

Fig. 6. Swelling kinetics of (1:2) ratio PEGD:CA/GEL and PEGD:GTA/GEL hydrogels at 25 °C and 37 °C: a) pH 5, b) pH 7 and c) pH 9.

Fig. 7. Pictures of the hydrogels degradation at different intervals of time. a-I) and a-II) correspond at PEGD:CA/GEL whereas b-I) and b-II) correspond at the PEGD:GTA/GEL, both hydrogels at 25 and 37 ºC respectively. The samples were exposed at phosphate buffered solutions (pH 7).

Fig. 8. In vitro drug release study at 37 ºC, a) physical crosslinked hydrogels PEGD:CA/GEL/TR at (1:0), (1:1) and (1:2) ratios; b) chemical crosslinked hydrogels PEGD:GTA/GEL/TR at (1:0), (1:1) and (1:2) ratios; SEM micrographs for c) PEGD:CA/GEL and d) PEGD:GTA/GEL.

Fig. 9. Suggested mechanism of Tramadol release.

Tables

Table 1. Hydrogel formulation.

EXPERIMENT (PEGD:AC)	PEGD 5 wt/vol% (mL)	CA-GTA 0.05M (mL)	GELATIN 25 wt/vol% (mL)	TRAMADOL (mg)
1:1	1	0.2	2	25
1:2	1	0.4	2	25
1:5	1	1	2	25

Table 2. Degradation time analysis of PEGD:CA/GEL and PEGD:GTA/GEL hydrogels in phosphate buffer pH 7 at 25 and 37 °C (average values).

PEGD:CA/GEL		PEGD:GTA/GEL	
25 °C	**37 °C**	**25 °C**	**37 °C**
90 h	15 h	180 h	20 h

Table 3. Kinetic parameters of the hydrogels with Citric Acid and Glutaraldehyde.

Kinetic parameters	PEGD:CA (1:1)	PEGD:CA (1:2)	PEGD:GTA (1:1)	PEGD:GTA (1:2)
n	0.94±0.08	0.86±0.051	0.96±0.281	0.74±0.023
k	0.63±0.005	0.73±0.004	0.95±0.005	0.70±0.002
R^2	0.98±0.018	0.99±0.006	0.96±0.018	0.98±0.056

Thermal and kinetic evaluation of biodegradable thermo-sensitive gelatin/poly(ethylene glycol) diamine crosslinked citric acid hydrogels for controlled release of tramadol

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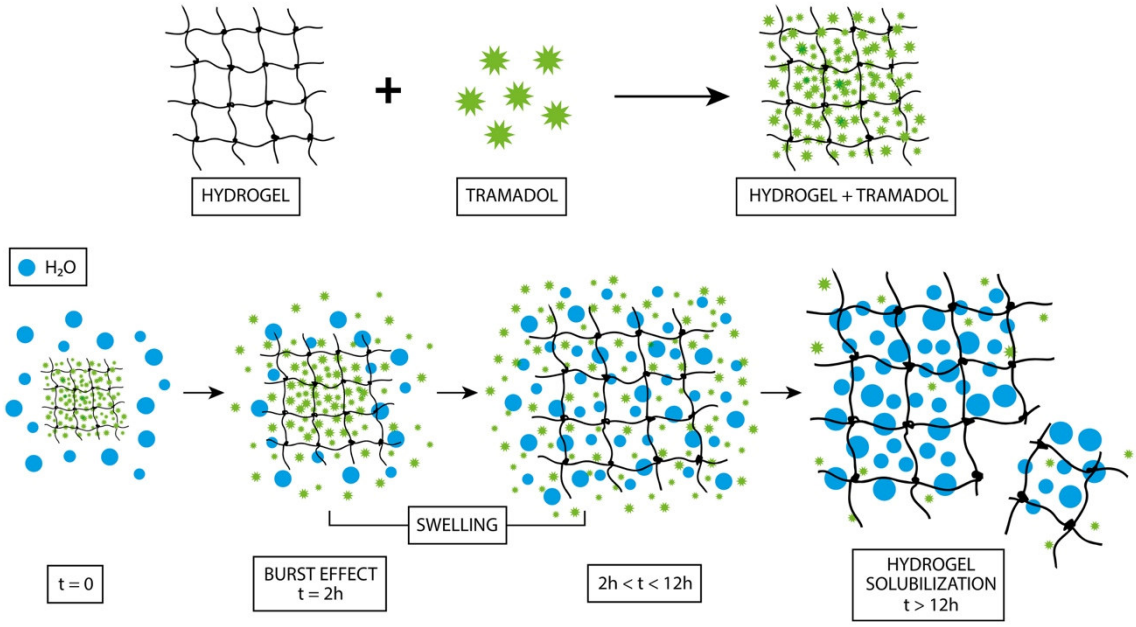

Highlights

- The properties of PEGD:CA/GEL can be tuned in terms of their application.
- The swelling behavior of PEGD:CA/GEL and PEGD:GTA/GEL is temperature-dependent.
- The addition of tramadol increases the mechanical properties of hydrogels.
- The drug release mechanism for PEGD:CA/GEL follows an anomalous mechanism.