Macromolecular Chemistry and Physic
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Hybrid Hydrogel Networks by Photocrosslinking of Thermoresponsive α,ω-Itaconyl-PLGA-PEG-PLGA Micelles in Water: Influence of the Lithium Phenyl-2,4,6-Trimethylbenzoylphosphinate Photoinitinator

Anna Zahoranova, Lucy Vojtova, Eva Dusicka, Lenka Michlovska, Nikola Krivankova, and Stefan Baudis**

Modification of thermogelling biodegradable copolymers with functional groups enables further covalent crosslinking of physical micelle-based hydrogels formed at specific temperature in water. The resulting hybrid hydrogel network exhibits an increase in stiffness and degradation stability. In this work, synthesized well-defined thermoresponsive α,ω-itaconyl-poly(D,L-lactide-*co*-glycolide)-*b*-poly(ethylene glycol)-*b*-poly(D,L-lactide-*co*-glycolide) (α,ω-itaconyl-PLGA-PEG-PLGA) macromonomers with a high degree of itaconyl-substitution providing free double bonds are photocrosslinked in water at both 25 and 37 °C using lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LiTPO) acting as water-soluble non-toxic photoinitiator. The effect of LiTPO on the thixotropic behavior of macromonomer in water at 25 °C without irradiation is evaluated. With the addition of a low amount of the photoinitiator (0.1 wt%), the degree of copolymer thixotropy increases. However, further increase in the photoinitiator concentration (0.5–3 wt%) leads to a lower degree of thixotropy. The photoinitiator is presumably interfering with the micellar self-assembly of the copolymer. This trend is also reflected in photocrosslinking efficiency, where the hybrid hydrogel networks with the highest storage moduli are achieved with very low concentrations of the photoinitiator (0.1 wt%) at 25 °C, while this trend is reversed at 37 °C. The hydrolytic stability of hydrogels prepared at 37 °C from 17 wt% macromonomer solution with 1% LiTPO exceeds 22 days.

1. Introduction

Thermogelling polymers are a class of stimuli-responsive materials that exhibit reversible gelation with the change of temperature. Especially interesting polymers are those, which form gels upon heating. Such materials can be used in various applications, such as food processing,[1] bioinks for 3D printing,[2] drug delivery applications,[3] and tissue engineering.[4]

Several natural as well as synthetic (co)polymers exhibit thermogelling behavior. While many naturally occurring thermogelling polymers, such as gelatin or agarose, form gel with decreasing temperature, different synthetic copolymers have been developed to exhibit opposite behavior. In principle, these are block copolymers with finely tuned hydrophilic/hydrophobic balance within the molecule. They can be composed of hydrophilic blocks, ensuring the hydration of the formed hydrogel, and thermoresponsive blocks, which collapse upon heating due to the hydrophobic interactions and are responsible for physical crosslinking. Alternatively, they possess hydrophobic blocks, enabling the self-assembly into micelles even below gelation temperature. With increasing temperature or concentration of the amphiphilic copolymer, the micelles pack into ordered structures, or aggregate to form networks.[5] The gelling temperature can be controlled by the length of the blocks, the ratio between

Dr. A. Zahoranova, Dr. S. Baudis
Institute of Applied Synthetic Chemistry
Vienna University of Technology
Getreidemarkt 9/163MC, Vienna A-1060, Austria
E-mail: stefan.baudis@tuwien.ac.at

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/macp.202000165.

DOI: 10.1002/macp.202000165

Prof. L. Vojtova, L. Michlovska, N. Krivankova
Central European Institute of Technology
Brno University of Technology
Purkynova 656/123, Brno 612 00, Czech Republic
E-mail: lucy.vojtova@ceitec.vutbr.cz

E. Dusicka
Polymer Institute of the Slovak Academy of Sciences
Dubravska cesta 9, Bratislava 845 41, Slovakia

hydrophilic and thermoresponsive parts, and the concentration of the copolymer in solution. Common examples used in biomedical applications are representatives of the poloxamer family, namely Poloxamers 407 and 403 among others, which are triblock ABA copolymers composed of hydrophilic poly(ethylene glycol) (A) and thermoresponsive poly(propylene glycol) (B). They have been extensively exploited for pharmaceutical applications.[4] Recently, Lorson and Luxenhofer have described a new thermogelling diblock copolymer composed of hydrophilic poly(2-methyl-2-oxazoline) and thermoresponsive poly(2-*n*-propyl-2-oxazine), which was successfully tested as bioink for biofabrication[2] and as a drug depot loaded with polymeric micelles for sustained release of curcumin.[3] However, the disadvantage of polyether- and poly(2-oxazoline)-based hydrogels is the lack of hydrolytically degradable groups, which would provide the possibility for controlled degradation in the human body.

An example of extensively used thermogelling copolymer containing degradable polyester groups is a triblock copolymer of poly(D,L-lactide-*co*-glycolide)-*b*-poly(ethylene glycol)-*b*-poly(D,L-lactide-*co*-glycolide) (PLGA-PEG-PLGA). In contrast to poloxamer copolymers containing outer hydrophilic blocks, this block copolymer contains hydrophilic inner blocks, which leads to different mechanism of gelation. In this case, the gelation is expected to proceed via aggregation of flower-like micelles and formation of irregular percolated network.[6] The commercial product named ReGel based on this copolymer has been developed and successfully tested for the delivery of various pharmaceutical compounds, such as paclitaxel (known as OncoGel), immunosuppressant cyclosporin A, insulin (known as ReGel), granulocyte colony-stimulating factor, porcine growth hormone, and recombinant hepatitis B surface antigen.[7,8] Clinical applications of ReGel have been summarized in recent review papers.[9,10] ReGel refers to copolymers with weight average molecular weight of 4200 Da, molar mass dispersity of about 1.3 and the molar ratio between D,L-lactide and glycolide of 3:1.[11] The degradation time of ReGel in vitro ranges from 6 to 8 weeks.[10] However, for certain applications, even longer degradation times are desirable. To achieve this, the covalent crosslinking of the copolymer can be employed in addition to physical one, to obtain a network with improved mechanical properties and slower degradation rates.

To introduce functional groups suitable for covalent crosslinking, Michlovská et al. have developed PLGA-PEG-PLGA copolymer end-functionalized with itaconic acid (*α,ω*-itaconyl-PLGA-PEG-PLGA), introducing thus both double bonds and carboxylic groups on both ends of polymer chain.[12] The itaconic acid is a monomer from renewable resources produced by *Aspergillus terreus*,[13] but is also secreted by activated mammalian macrophages.[14] It can be catabolized in mammalian liver mitochondria,[15] providing benefits over less biocompatible acrylates and methacrylates. The rheological properties and the thermoresponsive behavior of *α,ω*-itaconyl-PLGA-PEG-PLGA have been studied.[16] Functionalization with itaconic acid affected both the hydrolytical degradation and thermoresponsive properties of resulting copolymer.[17] The functionalization also leads to acceleration of degradation rate of the non-crosslinked copolymers at pH 7.4. The potential of covalent crosslinking for hydrogel preparation from *α,ω*-itaconyl-PLGA-PEG-PLGA macromonomer was tested.[18] The crosslinking reaction was performed only in bulk without additional crosslinker by the blue light with the wavelength between 430–490 nm with water-insoluble camphorquinone as a photoinitiator. However, the concentrated (bulk) macromonomer is very viscous and sticky (honey-like) avoiding precise dosing and manipulation.

In this study, we aimed to extend the above mentioned work[18] and decided to explore the potential of covalent crosslinking of *α,ω*-itaconyl-PLGA-PEG-PLGA in aqueous solutions in order to extend its medical application to injectable and sprayable delivery systems. In this case, the situation is more complicated, since the temperature-driven self-assembly of the copolymer in solution (i.e., formation of micelles or aggregates) has to be considered. As photoinitiator we selected lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LiTPO) due to its sensitivity to visible light. Moreover, it was proven to possess superior water-solubility and comparable biocompatibility to the commonly used Irgacure 2959.[19] First, we characterized the thermoresponsive behavior of aqueous solutions of *α,ω*-itaconyl-PLGA-PEG-PLGA macromonomers at three different concentrations, 9, 13 and 17 wt%, with the emphasis on gelation kinetics in the range from 25 to 50 °C. Further, the effect of LiTPO phototinitiator on the thixotropic behavior of the block copolymer was studied in detail to unravel the microstructural changes in the copolymer solutions. Then, we performed chemical photocrosslinking at both 25 and 37 °C in order to find an optimal concentration of the photoinitiator to achieve improved mechanical properties of the hydrogels.

2. Results and Discussion

2.1. Synthesis of *α,ω*-Itaconyl-PLGA-PEG-PLGA

The copolymer *α,ω*-itaconyl-PLGA-PEG-PLGA was synthesized by one-pot two-steps synthesis as previously described by Michlovská et al.[12] In the first step, the triblock copolymer PLGA-PEG-PLGA was prepared by ring-opening polymerization, starting from PEG ($\bar{M}_n$ = 1500 g mol^{-1}) as a macroinitiator, with the LA (D,L-lactide) to GA (glycolide) molar ratio of 3.0 and PLGA to PEG weight ratio of 2.5. In the second step, the copolymer was modified with itaconic anhydride. The product was analyzed by ^{1}H NMR and GPC measurements. The ^{1}H NMR spectrum of the sample is shown in **Figure 1**. The obtained spectrum corresponds well with previously described syntheses.[12,17,18] Number-average molecular weight $\bar{M}_n$ and molar composition of macromonomer sample were determined from integrals of characteristic proton intensities of PEG (OCH_2CH_2O) at δ = 3.55–3.75 ppm (multiplet, 4*H*) (1), lactic acid ($O(CH_3)CHO$) in a range between δ = 5.1–5.35 ppm (multiplet, 1*H*) (3), and ($OC(CH_3)CHO$) protons at δ = 1.5–1.75 ppm (multiplet, 3*H*) (4) and glycolic acid (OCH_2O) at δ = 4.6–4.9 ppm (multiplet, 2*H*) (5). The amount of end-capped ITA was determined from integrals of characteristic proton intensities of itaconic acid backbone ($OCH_2(C=O)$) at δ = 3.40–3.44 ppm (singlet, 2*H*) (6) and itaconic acid double bonds ($OC(CH_2)CCH_2COOH$) at δ = 6.35–6.5 ppm (singlet, 1*H*) (7a) and at δ = 5.7–5.8 ppm (singlet, 1*H*) (7b). The $\bar{M}_n$ of macromonomer calculated from

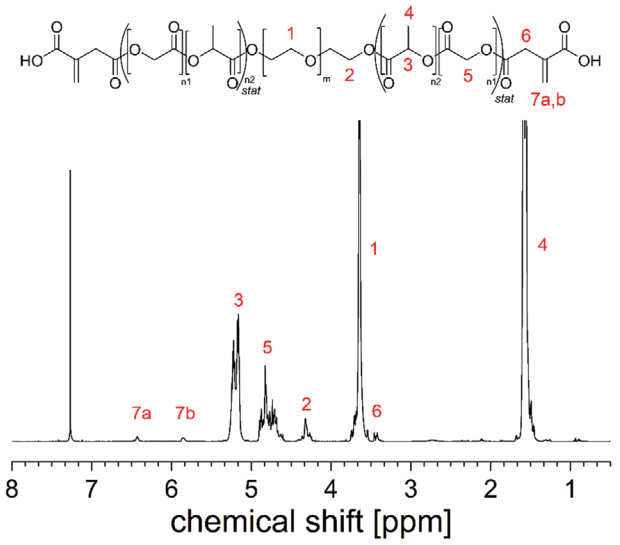

Figure 1. ^{1}H NMR spectrum of α,ω-itaconyl-PLGA-PEG-PLGA.

^{1}H NMR spectra (5280 g mol^{-1}) was in very good agreement with theoretical $\bar{M}_n$ (5280 g mol^{-1}) and the $\bar{M}_n$ determined by gel permeation chromathography with multi-angle light scattering method (GPC-MALS) (5580 g mol^{-1}). Final molar mass dispersity (1.19) was very narrow proving living character of ring-opening polymerization. From the NMR spectrum, the molar ratio of LA to GA equal to 2.96 and the weight ratio of PLGA to PEG equal to 2.52 were calculated, which is in good accordance with the values from the feed. Since the copolymer was planned to be used for further chemical crosslinking, the high degree of ITA modification (having value of 89 mol%) was desirable. As previously shown in the work of Michlovská, et al.,[12] the degree of modification of α,ω-itaconyl-PLGA-PEG-PLGA is affected by purification of itaconic anhydride (ITA) by sublimation, since presence of moisture causes transition of itaconic anhydride to itaconic acid, leading to undesired side reactions. In this work, we changed the ITA distributor, purchasing it with higher purity (98% comparing to 95%). Moreover, we improved bulk mixture stirring enabling higher degree of modification of our copolymer giving aforementioned 89 mol% in comparison to previously published 63 mol%.[18] Since the functionalization with itaconic anhydride was shown to affect the properties of copolymer, such as thermoresponsive behavior and hydrolytic stability,[17] we assumed that the behavior of our copolymer can be slightly different in comparison with previously described systems with lower degree of modification.

2.2. Thermoresponsive Behavior of α,ω-Itaconyl-PLGA-PEG-PLGA

We characterized thermoresponsive viscoelastic behavior of α,ω-itaconyl-PLGA-PEG-PLGA copolymer in water, since we assumed that the physical gelation of copolymer caused by the temperature increase is expected to have an effect on the chemical gelation. The dependence of storage (G') and loss moduli (G'') on temperature for three selected concentrations of the copolymer in water, that is, 9, 13, and 17 wt% is depicted in **Figure 2**. Increasing temperature and concentration induce the structural changes of micelles accompanying by change in turbidity of solution.

With the increase of temperature, the copolymer solutions exhibit two intercept points of G' and G''. At room temperature, G'' exceeds G' for all copolymer concentrations, corresponding to the sol-state of the samples. At all prepared concentrations, polymer chains form micelles as assumed from similar studies dealing with the unmodified copolymer.[21] The micelles are amphiphilic having the hydrophobic PLGA "core" and hydrophilic PEG shell. The hydrophobic PLGA blocks minimize free energy in water and hence a compact core is formed, surrounded by hydrophilic PEG blocks. The first intercept point is related to the sol–gel transition temperature (T_{SG}). With the further increase of the temperature, the second intercept cloud-point appears, which is related to the gel–sol transition (T_{GS}) caused by the dehydration of PEG headgroup resulting in packing the micelles into the large irregular aggregates clouding the solution. The occurrence of cloud point upon heating is a phenomenon generally observed in non-ionic surfactant systems caused by attractive interactions between non-ionic micelles or by the transition from linear to branched micelles.[22] The characteristic values obtained from these heating curves are summarized in Table S1, Supporting Information. As expected, the concentration of the copolymer affects the stiffness of the gels. With increasing concentration, G'_{max} is rising faster due to the higher amount of entangled micelles. On the other hand, the concentration influences the transition temperatures of the samples only very little. The T_{SG} was 30.9 °C for 9 wt% copolymer solution. With the increasing concentration, the T_{SG} slightly decreased 30.2 ± 0.4 °C for 17 wt% solution.

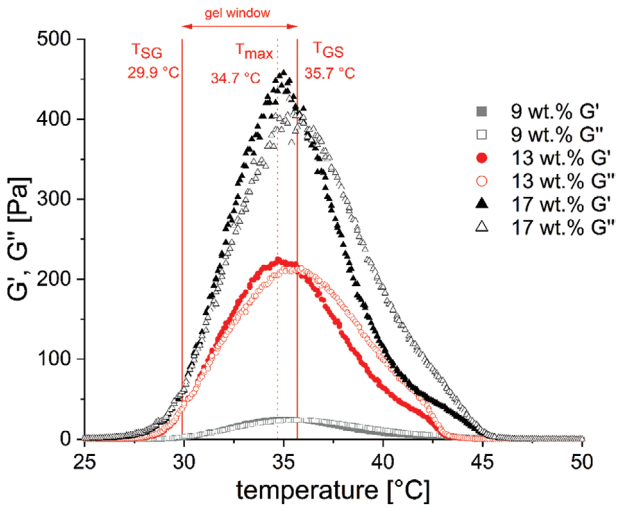

Figure 2. Thermoresponsive behavior of α,ω-itaconyl-PLGA-PEG-PLGA at three different concentrations in water. The selected dependences of storage modulus (G′, filled symbols) and (G″, empty symbols) of different concentrations of copolymer in water (9 wt%—gray squares, 13 wt%—red circles, 17 wt%—black triangles). The sol–gel transition temperature (T_{SG}, the temperature of the first intercept point of G′ and G″), the gel–sol transition temperature (T_{GS}, the temperature of the second intercept of G′ and G″) and T_{max} (the temperature of the maximal G′) are shown for the concentration 13 wt%.

The T_{GS} values are also quite similar, they range from 35.9 °C for 9 wt% solution to 36.0 ± 0.2 °C for 17 wt% solution. The temperature-driven gelation of α,ω-itaconyl-PLGA-PEG-PLGA or related copolymers is affected by various parameters, such as molar mass of the copolymer, the ratio of PLGA to PEG, the ratio of D,L-lactide to glycolide (LA/GA), or the degree of substitution of ITA.[16,17,23]

It should be emphasized that this characterization of thermoresponsive behavior of the copolymer was performed under the standard conditions, where the copolymer solutions were kept on ice prior the measurements and heated with the rate of 0.5 °C per minute (for more details, see Experimental Section: Rheological measurements). This is a commonly accepted procedure for the characterization of thermogelling polymers.[2,24,25] However, in this work different behavior was observed, when the copolymers in aqueous solutions were kept at room temperature for prolonged time (i.e., 1–2 days). In that case, physical gel formed for all the copolymer concentrations, even though the temperature was below the T_{SG} (Figure S1, Supporting Information). To understand this behavior, we studied the gelation kinetics at 25 °C in more detail. The changes of the storage and loss modulus with time at 25 °C for the copolymer concentration 13 wt% are depicted in Figure S2, Supporting Information. The both moduli are gradually increasing with time, with the intercept point reached after 108 min. A similar behavior was recently reported for Pluronic F127 (poloxamer 407) thermogelling hydrogels, where the long relaxation times during rheological measurement near the T_{SG} were measured.[26] Such long relaxation times were attributed to the formation and relaxation of large clusters of micelles, rather than the formation and reorganization of individual micelles, since these are fast processes within the time scale of 100 ms. This explanation is also applicable to our system, where the micelles aggregation is accompanied by the visual observation of opaque physical gel. Indeed, the internal structure of PLGA-PEG-PLGA thermogels was described as an irregular percolated network of micellar aggregates, with the size of the aggregates in the range of visible light wavelengths.[6] The presence of irregular network was further supported by Nyström et al. using the small angle neutron scattering.[21] It should be noted that in previous works on rheological properties and thermogelation of α,ω-itaconyl-PLGA-PEG-PLGA, or in other work on unmodified PLGA-PEG-PLGA copolymers, the gelation kinetics were not studied, and the physical gelation of the copolymer after the prolonged incubation at the temperature below T_{SG} has not been described so far.

2.3. Effect of LiTPO on the Thixotropy of α,ω-Itaconyl-PLGA-PEG-PLGA

After the study of physical crosslinking of α,ω-itaconyl-PLGA-PEG-PLGA, we focused on the covalent crosslinking, exploiting the end-functionalization of the copolymer with double bonds. For covalent crosslinking of the copolymers by irradiation, the addition of a suitable photoinitiator is necessary. In our work, we selected the photoinitiator LiTPO, due to its favorable water-solubility, biocompatibility, and absorbance in the blue part of visible light region. First, we aimed to study the effect of LiTPO

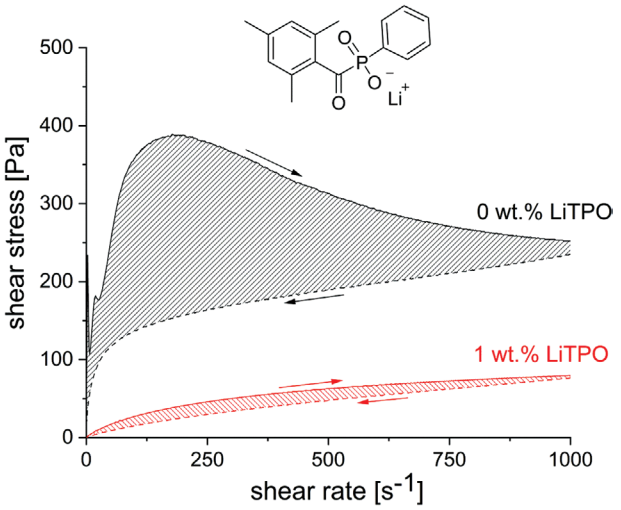

Figure 3. Representative dependences of the shear stress on the increasing and decreasing shear rate (marked by arrows) for 17 wt% of the copolymer α,ω-itaconyl-PLGA-PEG-PLGA in water without the addition of photoinitiator LiTPO (black curves) and with the addition of 1 wt% of the photoinitiator LiTPO (red curves). The areas between the up and down curves (the hysteresis loops) are related to the thixotropic behavior.

on the physical interactions between the copolymer chains in aqueous solution. For this purpose, we measured the shear stress for continuously increasing and decreasing shear rate for different concentrations of the LiTPO. The experiment was done under light protection thus no covalent crosslinking was expected. The representative curves for 17 wt% of copolymer aqueous solution without the LiTPO and with the addition of 1 wt% of LiTPO are depicted in **Figure 3**. For the solution with 17 wt% of copolymer without the photoinitiator, the curves for increasing and decreasing shear rates differ, forming a hysteresis loop. This effect is one of the typical manifestations of thixotropic behavior.[27] The thixotropic behavior was observed for various types of samples, including mineral slurries,[28] blood,[29] or paints.[30] Certain solutions containing micelles, including thermogelling poloxamers, were also reported to exhibit thixotropic behavior.[31,32] In the recent paper of Vojtová et al., the authors have studied the impact of PLGA-PEG-PLGA copolymer on rheological properties of tricalcium phosphate cement for bone regeneration.[33] For the block copolymer solution without the filler, they observed only small hysteresis in shear stress versus shear rate plot. However, in that case, the maximum shear rate used was 100 s^{-1}, which was lower than in case of the current work. It should be noted that the thixotropic behavior of our samples can be beneficial for the further development of injectable hydrogel formulations for in vivo applications.

After the addition of 1 wt% of the photoinitiator, the hysteresis loop diminishes, indicating that the addition of the photoinitiator affects the self-assembly behavior of the micelles, leading to the disruption of self-assembled structures. To study this effect in detail, we plotted the area under the hysteresis for the three different concentrations of the copolymer (9, 13, and 17 wt%) and four different concentrations of the photoinitiator (0.1, 0.5, 1, and 3%) in **Figure 4**. The area under the hysteresis can be considered as a relative parameter connected to the degree of thixotropy, which means the degree of structural

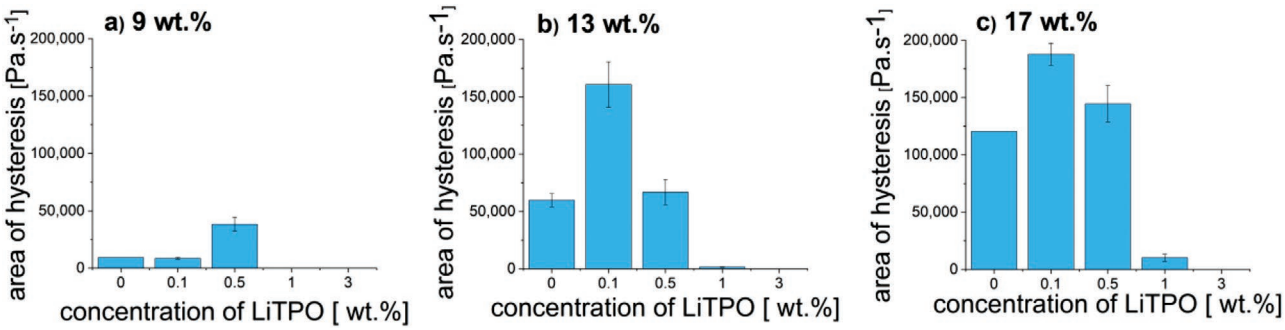

Figure 4. Influence of the concentration of the photoinitiator on the area of the hysteresis loop of up and down shear stress/shear rate curves for a) 9 wt%, b) 12 wt%, and c) 17 wt% of the copolymer in water measured at 25 °C without irradiation. The values are depicted as the mean ± SD from three measurements. The values for 1 and 3 wt% of LiTPO for 9 wt% of the copolymer and 3 wt% of PI for 13 and 17 wt% of the copolymer were too low to be displayed.

breakdown under the shear.[27,34] It can be seen that after the addition of low concentrations of photoinitiator (0.1 wt% of LiTPO for 13 and 17 wt% copolymer solution, 0.5 wt% of LiTPO for 9 wt% of the copolymer solution), the area under the hysteresis increased for all the concentrations of copolymer. However, with the further increase of the photoinitiator concentration, the area under the hysteresis starts to decrease, depending also on the concentration of the copolymer. For the lowest concentration of the copolymer, 9 wt%, the hysteresis loop is completely diminished for 1 wt% of the added LiTPO. For the higher concentrations of copolymer, 3 wt% of the LiTPO is necessary to completely suppress the thixotropic behavior. This is probably due to the higher number of self-assembled structures.

In order to explain this behavior, we investigated how the addition of LiTPO affects the properties of the sample solutions. We studied the effect of the LiTPO addition on the pH of the copolymer solutions, since the pH value can affect the degree of deprotonation of carboxyl end groups. The presence of a charge at copolymer chains would lead to the increased repulsive forces and disruption of self-assembled structures. The dependence of pH of the solution on the content of LiTPO is depicted in Figure S3, Supporting Information. It should be noted that even though the addition of LiTPO indeed led to the increasing pH of the copolymer solutions, the resulting pH even after addition of 3 wt% of photoinitiator is below the acid dissociation constant pK_a of carboxylic acid from itaconyl group, which is 3.85. However, as it was shown for poloxamer end-capped with carboxyl groups, the dissociation constant of micelles pK_m is lower than that of free polymer chains in solutions pK_a.[35] We assume a similar behavior for α,ω-itaconyl-PLGA-PEG-PLGA. On the other hand, the change of the pH is not so prominent, especially for low concentrations of added LiTPO. As an example, the difference in pH between 0 and 0.1 wt% of the LiTPO in case of 13 wt% of copolymer is around 0.01. This negligible change could not explain the sharp increase of hysteresis loop area in thixotropic curves. Another parameter affected by the addition of LiTPO is the ionic strength of the solutions. The increased ionic strength was shown to dramatically decrease the cloud point of both unmodified poloxamer and poloxamer modified with carboxyl groups.[36] In addition, triblock copolymer PEG-PLGA-PEG exhibited sharp decrease of gel temperature, 10 °C upon the addition of 3 wt% of NaCl, due to the salting-out effect.[37] Since the lithium cations are kosmotropic, we could also expect a salting-out effect, which can cause the aggregation and phase separation of the micelles. In conclusion, the effect of LiTPO on the self-assembly of the copolymer is expected to be a complex process combining several effects, leading to non-linear dependencies as shown in Figure 4. The initial increase of hysteresis loop can be attributed to the charge shielding effect of lithium cations on partially deprotonated carboxyl groups, the subsequent thixotropy suppression with increasing concentration of the LiTPO is assumed to be due to the effect of increased pH on deprotonation of the itaconyl groups, or the effect of increased salt concentration on aggregation behavior of thermoresponsive polymer backbone.

2.4. Covalent Crosslinking of α,ω-Itaconyl-PLGA-PEG-PLGA at 25 °C

In the previous section, we have shown that the addition of the negatively-charged photoinitiator affects the self-assembly behavior of the copolymer, manifested in changes in thixotropic behavior of the samples. We assumed that this behavior will also be reflected in the crosslinking efficiency during the irradiation. To test this assumption, the photorheological measurements at both 25 and 37 °C were performed. It should be noted that even though the temperature 25 °C is associated to the sol-region of the phase diagram of the copolymer (see Figure 1), all the copolymers (without the photoinitiator) were in the gel phase even before the start of irradiation as shown in Figure S2, Supporting Information. This was caused by the prolonged incubation of the samples at room temperature before measurements, which triggered the physical gelation (see the section 2.1). The development of the storage modulus during the photocrosslinking of the copolymers with concentration 17 wt% with different amounts of photoinitiator is depicted in **Figure 5**. Both storage and loss moduli are shown in Figure S4, Supporting Information. The control sample without the LiTPO does not exhibit increase in storage modulus after the start of the irradiation (time point 900 s), on the contrary, the modulus slightly decreased. This slight decrease of the modulus was observed for all the concentrations of the copolymer and is probably caused by local temperature inhomogeneities caused by the irradiation. After the start of irradiation, the storage modulus of all the sam-

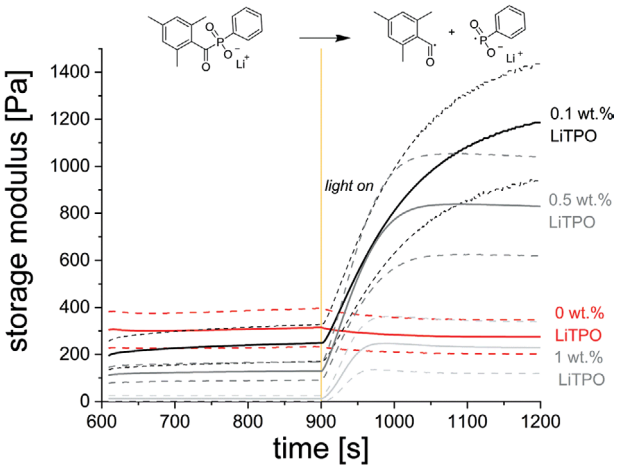

Figure 5. Evolution of the storage modulus (G′) of the 17 wt% copolymer in water with the different concentrations of the LiTPO during the irradiation at 25 °C. The lines are displayed as an average and 95% confidence interval (dashed lines) from three measurements.

ples containing photoinitiator increased, which indicates the covalent crosslinking process. The crosslinking process is expected to proceed via radical attack of the double bond of the itaconyl end group, followed by the polymerization reaction, as described in details by Michlovská et al.[18] Interestingly, storage modulus increases with decreasing amount of photoinitiator in the sample, with the highest modulus obtained for 0.1 wt% of the photoinitiator. This finding is in accordance with the observed changes in hysteresis loop area discussed in the previous section, where the area under the loop is decreasing with increasing percentage of LiTPO. We explained these changes as a disruption of the self-assembled structures of copolymer by the high amount of LiTPO. The photorheological experiments indicate that after this disruption of the internal self-assembled structure, the ability copolymers to form covalently crosslinked network in the aqueous solution is hindered, since a part of itaconyl end groups is no longer in proximity caused by suppressed self-assembly. However, it should be noted that we observed increase in storage moduli after irradiation in all cases (except the control without the LiTPO), indicating that a fraction of the copolymer is still able to form covalently crosslinked networks. This impact of the distance between reactive acrylate groups on the crosslinking rate of micellar hydrogels was also described by Jabbari et al.[38] It was also shown that the addition of thermogelling poloxamer 407 into PEG-diacrylate leads to the faster formation of covalently crosslinked network, presumably due to the local higher concentration of acrylate groups in aqueous phase.[39]

To compare the mechanical properties of hydrogels prepared by covalent crosslinking, we plotted the maximal storage modulus achieved during the photocrosslinking (G'_{max}) for all types of tested hydrogels (**Figure 6**). For the comparison, we added also the values for 0 wt% of LiTPO (the physical gel formed after 3 days of incubation at 25 °C) and thermogel (maximum value of G' obtained during the heating of the sample, see Table S1, Supporting Information). For all concentrations of copolymers, the highest G'_{max} were achieved for the concentration of LiTPO 0.1 wt%. For all the concentrations of the copolymer, the modulus G'_{max} decreased with the increasing concentration of the photoinitiator, the same trend as described in the previous paragraph for 17 wt% of the copolymer. For the two highest concentrations 13 and 17 wt%, this trend is in line with the relation to the area under hysteresis curve, as discussed above. That is, the decrease of the area under the hysteresis correlates with the decrease of the G'_{max} for increasing concentration of LiTPO. However, for the lowest concentration, 9 wt%, similar trend is not observed. This can be also due to the low absolute values and differences of G'_{max} for 9 wt% copolymer concentration, where the correlation between G'_{max} and the area under the hysteresis are not so pronounced. For better illustration, the correlation between G'_{max} and area under the hysteresis is depicted in Figure S5, Supporting Information.

For 1 wt% LiTPO, the G'_{max} reached the comparable or even lower value as the physical gel (0% LiTPO). The G'_{max} for the hydrogels made with 0.1 wt% of LiTPO exceed the G' values of the thermogels in all concentrations of the copolymer. For illustration, G'_{max} of 17 wt% of copolymer with 0.1 wt% reaches the value 1233.3 ± 130.1 Pa, which is 2.3-fold increase in comparison to G' value of the thermogel (530 ± 3 Pa). This finding proves that the additional covalent crosslinking by irradiation at 25 °C improves the mechanical properties of α,ω-itaconyl-PLGA-PEG-PLGA hydrogels. This crosslinking efficiency

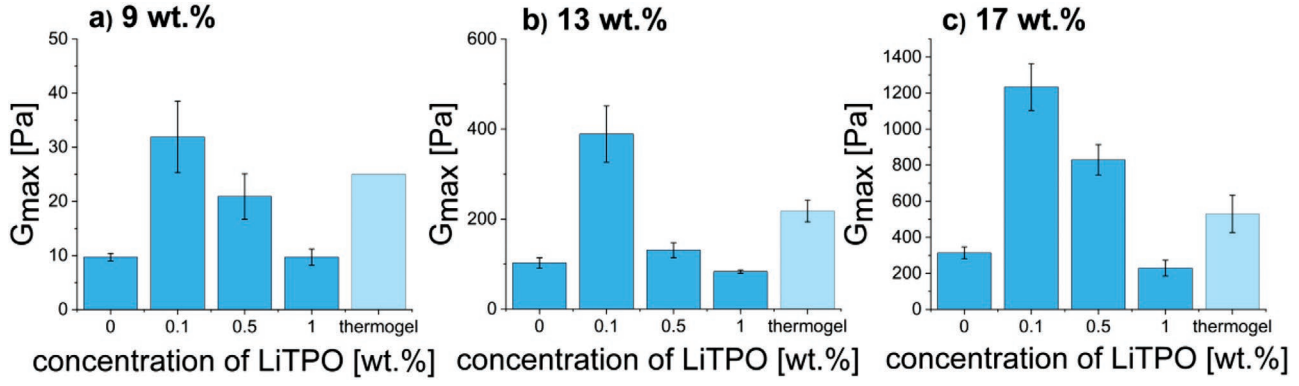

Figure 6. Maximum storage modulus (G'_{max}) of irradiated hydrogels with different percentage of photoinitiator. For the comparison, G'_{max} value of hydrogel physically crosslinked by heating is shown. The values are displayed as mean ± SD from three measurements. Please note that the scale on y axis is different for different concentrations of copolymer.

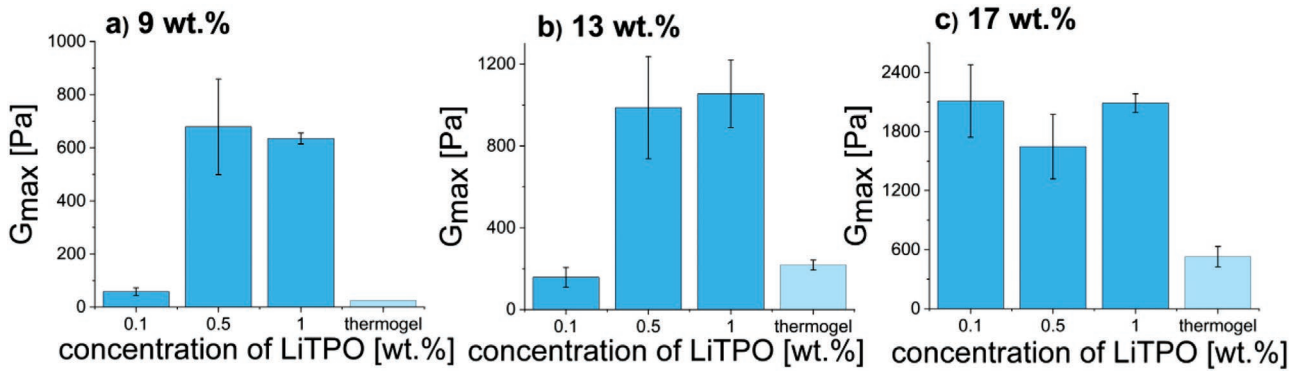

Figure 7. Maximum storage modulus (G'_{max}) of hydrogels irradiated at 37 °C with different percentage of photoinitiator. The values are displayed as mean ± SD from three measurements. Please note that the scale on y axis is different for different concentrations of copolymer.

increases with the polymer concentration providing a larger amount of reactive agglomerated micelles.

2.5. Covalent Crosslinking of α,ω-Itaconyl-PLGA-PEG-PLGA at 37 °C

However, for the medical application of α,ω-itaconyl-PLGA-PEG-PLGA hydrogels, for example, as drug/protein delivery systems in the treatment of wounds, photocrosslinking of thermogels at 37 °C is important.

The development of the storage modulus during the photocrosslinking of the copolymers with concentration 9 wt% with different amounts of photoinitiator at 37 °C is depicted in Figure S6, Supporting Information. All samples were already in a gel state before the irradiation where G' was above G''. After 300 s the light was triggered on for different time, dependent on the duration of modulus increase.

In **Figure 7** the comparison of G'_{max} for all measurements at 37 °C is shown. It is obvious that the higher the macromonomer content the higher G'_{max} was observed. However, comparing to photocrosslinking at 25 °C, G'_{max} was either negligible or very low when applying 0.1 wt% of LiTPO in 9 and 13 wt% polymer solution, respectively. Both weak micellar network and low photoinitiator concentration suppress chemical crosslinking at these conditions. However, with increasing macromonomer concentration, the amount of LiTPO has lower influence on G'_{max}. For example, very high modulus (approximately from 1500 to 2100 Pa) and best mechanical properties were achieved for 17 wt% macromonomer solutions photocrosslinked at 37 °C using either low or high concentration of LiTPO (0.1–1 wt%). Nevertheless, the increase of the modulus took very long time especially for 0.5 and 1 wt% of LiTPO at 37 °C (≈85 min) in comparison to 25 °C (≈20 min). It was assumed that at 37 °C the physical gel was stronger before the irradiation, micelles were more connected and aggregated between each other and therefore the radical propagation through double bonds in the middle of micelles took considerably longer.

Since it is known that photocrosslinking of physical network increase the hydrogel's hydrolytic degradation, the 17 wt% copolymer solution has been irradiated at 37 °C with 1% LiTPO and kept in ultra-pure water at 37 °C (see Figure S7, Supporting Information). Prepared gels were stable longer than 22 days, comparing to the physical thermogel of the same α,ω-itaconyl-PLGA-PEG-PLGA macromonomer with similar concentration (16 wt%), which has not exceeded 11 days either in pH 4.3 or 7.4.[17] For the comparison, swelling ratio of hybrid hydrogel in physiological solution at 37 °C was monitored during 20 days to assess hydrolytic stability of the hybrid hydrogel samples (Figure S8, Supporting Information). For this study, a new batch of the copolymer was used. The hybrid hydrogels achieved maximum swelling ratio 11.8 ± 2.5 after 3 days, followed by decrease in swelling ratio due to the degradation. The hybrid hydrogels dissolved after 20 days under studied conditions.

3. Conclusions

In this paper, we presented for the first time the hybrid hydrogel network made by both physical and covalent photocrosslinking of the α,ω-itaconyl-PLGA-PEG-PLGA macromonomer in aqueous environment, using LiTPO as a photoinitiator. We have proven that additional covalent crosslinking by irradiation leads to increased storage moduli for all tested concentrations of the copolymer at both 25 and 37 °C. In addition, we focused on the influence of concentration of the added photoinitiator on self-assembly behavior of the copolymer. Although the photoinitiator LiTPO is gaining an increasing attention especially in biomedical applications,[40,41] the effect of the concentration of this photoinitiator on the self-assembly and photogelation of thermoresponsive (co)polymers seems to be an overlooked problem in the scientific community.[42–44] In this paper, we have shown that the addition of low amount of the photoinitiator (0.1 wt%) led to improved formation of self-assembled structures at 25 °C, manifested by increased area of the hysteresis curve. This behavior is also reflected in the formation of photocrosslinked network with highest storage modulus. Although with increasing amount of the photoinitiator, the hysteresis loop, as well as the photogelation ability of the copolymers at 25 °C decreases, the opposite situation was observed at 37 °C, where the LiTPO concentration effect disappears with increasing macromonomer (micelles) concentration. Here, we described, how to affect hydrogel network

mechanical properties by different conditions like temperature and concentration of both macromonomer and photoinitiator. Hydrogel network properties (stiffness, density, etc.) influence hydrogel hydrolytic stability and water diffusion going along with controlled drug/protein release useful in disease treatment or regenerative medicine. Similar effects as described in our work could be also expected for other thermoresponsible photocrosslinkable amphiphilic copolymers.

4. Experimental Section

Materials: LiTPO was synthesized as described in the work of Benedikt et al.,[19] D,L-lactide (LA, ≥99.9%) and glycolide (GA, ≥99.9%) were supplied by Polysciences (Pennsylvania). Poly(ethylene glycol) (PEG, $\bar{M}_n = 1500$ g mol^{-1}), Sn(II)2-ethylhexanoate (95%) were purchased from Sigma-Aldrich (Germany). Itaconic anhydride (ITA, 98%) was purchased from Acros Organics. Ultrapure water (Type 1 according to ISO 3696) was prepared using Millipore purification system (MilliQ Academic, Millipore, France).

Synthesis of α,ω-Itaconyl-PLGA-PEG-PLGA: Thermosensitive PLGA-PEG-PLGA triblock copolymer with molar ratio of LA/GA equal to 3.0 and weight ratio PLGA/PEG equal to 2.5 was synthesized under nitrogen atmosphere via ring opening polymerization method in a bulk according to Michlovská et al.[12] The copolymer was functionalized with ITA (2.5 mol ratio) under nitrogen atmosphere and intense stirring at 110 °C for 1 h. In order to separate the prepared copolymer from unreacted monomers, the product was dissolved in ultra-pure water (≈10 wt%) at 8 °C. The solution was then heated to 80 °C and the copolymer precipitated from the solution. The solution with the dissolved monomers was disposed. The decantation was repeated three times. The product was freeze-dried (Martin Christ lyophilizator EPSILON-2D) until constant weight (yield 83%) and stored in sealed containers under pure nitrogen atmosphere to prevent hydrolysis.

Preparation of Aqueous Solutions of α,ω-Itaconyl-PLGA-PEG-PLGA: α,ω-Itaconyl-PLGA-PEG-PLGA was weighed into a vial and calculated amount of the ultra-pure water was added. After the addition of water, samples were stirred for 3–5 days at 8 °C, until a homogeneous solution was obtained. Solutions were prepared with three different concentrations of macromonomer, 9, 13, and 17 wt%. Prior to photorheological measurements, the samples were stored at ambient conditions for 2 days. Calculated amount of LiTPO photoinitiator (PI) (0.1, 0.5, 1, or 3 wt%) was added directly to the macromonomer aqueous solution before the photorheological measurement. After the addition of PI, the samples were handled under light protection in a yellow light lab, where wavelengths below 480 nm are filtered (using adhesive foils from the company IFOHA to cover windows and fluorescent lamps). Before every measurement the samples were vortexed to assure homogeneity.

Analytical Methods: Molecular weight determination and polymer characterization were performed using proton nuclear magnetic resonance ^{1}H NMR spectroscopy on 700 MHz Bruker AVANCE III HD instrument using 128 scans in deuterated chloroform (CDCl3) at 25 °C. Chemical shifts were reported in ppm relative to tetramethylsilane. ^{1}H NMR spectra were evaluated using ACD/1D NMR Processor.

Number-average molecular weight ($\bar{M}_n$) and molar mass dispersity ($Đ = \bar{M}_w/\bar{M}_n$) of the α,ω-itaconyl-PLGA-PEG-PLGA macromonomer were determined using GPC-MALS. An instrumental setup included Agilent HPLC 1 100 Series instrument with degasser, pump, autosampler, set of two PLgel 5 mm Mixed-C 300 × 7.5 mm columns (Agilent, USA) tempered to 25 °C and UV–vis diode array detector in connection with a DAWN HELEOS II multi-angle laser light scattering detector, ViscoStar-II differential viscometer, and Optilab T-rEX refractive index detectors (Wyatt Technology, Germany). Both MALS and RI detectors operated at 658 nm. THF was used as the mobile phase at a flow rate of 1 mL·min^{-1}. Astra 6.1 software was used for data collection and analysis.

Rheological Measurements: Modular Compact Rheometer Anton Paar Physica MCR 300 equipped with Peltier temperature control was used for the measuring of shear rate sweeps, temperature, and time sweeps. Shear rate sweeps were performed in plate to plate configuration (diameter 25 mm) with the constant gap 0.15 mm. Cone-plate configuration was used to measure temperature and time sweeps. Upper cone plate with the diameter 25 mm and cone angle 0.979° and band gap 48 µm was used.

Shear rate sweeps measurements were carried out at 25 °C for aqueous solutions. Shear rate was linearly increased from 1 s^{-1} to 1000 s^{-1} in 500 s, followed by 10 s equilibration at 1000 s^{-1} and subsequent decrease to 1 s^{-1} in 500 s. Temperature sweeps of 9, 13, and 17 wt% copolymer solutions from 8 to 60 °C were performed. Measurements were performed at shear stress 0.4 Pa and angular frequency 1 rad s^{-1}. The rate of heating was 0.5 °C min^{-1}. 150 µL of the sample stored in an ice bath was pipetted on the plate pre-cooled to 8 °C. At the trim position, excess of the formulation was wiped off with a paper tissue. Afterward, paraffin oil was applied on edges of the steel stamp at the measuring position to avoid evaporation.

Photorheological Measurements: Photorheological measurements were carried out with Anton Paar Modular Rheometer MCR 302 WESP. The detailed set-up description is presented in the work of Gorsche et al.[20] Plate-to-plate configuration with the diameter 25 mm was used. Bottom immobile glass plate enables light irradiation. As the light source Omnicure type S2000-XLA with the range of wavelengths 400–500 nm with the intensity 4 W·cm^{-2} at the tip of the light guide was used to irradiate aqueous solutions. Omnicure was calibrated by EXFO R2000 Radiometer.

Samples were applied in the middle of the glass plate with a spatula. Afterward, the steel stamp was lowered to the measuring position. Excess of sample was wiped off by paper tissue. Liquid samples were applied on the glass plate by micropipette (75 µL) directly into the measuring gap. Subsequently, the paraffin oil was applied on edges of the steel stamp before every measurement of aqueous solution to avoid the evaporation. Measurements were performed with a constant gap of 150 µm between measuring plates. Amplitude of the strain and frequency for all measurements were set to 1% and 1 Hz, respectively. G' and G'' were measured every second during the experiment.

Samples measured at 37°C were applied on the glass plate at 25 °C before the start of oscillation. Afterward, increase in temperature from 25 to 37 °C proceeded at a rate 2 °C min^{-1} followed by 20 min samples relaxation at 37 °C.

Statistics: The (photo)rheological measurements were performed on triplicates from one formulation to ensure reproducibility. The results are displayed as mean ± SD, if not stated otherwise. The photorheology curves are displayed as mean ± 95% confidence interval from three measurements.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

A.Z. and E.D. contributed equally to this work. This research was funded by the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II under the project CEITEC 2020 (LQ1601). A.Z. would like to acknowledge Austrian Science Fund (FWF): M 2805-N (Meitner Programm) for financial support.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

injectable hydrogels, photocrosslinking, photoinitiators, thermogellation, thixotropy

Received: May 12, 2020
Revised: June 23, 2020
Published online: August 9, 2020

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